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Abstract

Due to only few experimental evidences, the importance of root-induced alteration of metal dynamic speciation in the rhizosphere in the determination of metal bioavailability to plants is still a matter for debate. The present study thus investigated how root-induced changes in pH and dissolved organic matters (DOM) altered copper (Cu) dynamic speciation in the rhizosphere of durum wheat (*Triticum turgidum durum* L.). Plants were exposed to a Cu-contaminated soil previously alkalised by liming to cover soil pH values ranging from 4.8 to 7.5. A range of analytical techniques was deployed on soil exposed (i.e. in the rhizosphere) or not (i.e. in the bulk soil) to plant roots, including the measurement and the modelling (using the Humic Ion-Binding Model VI) of Cu^{2+} activity, the measurement of labile Cu concentration and Cu lability by Differential Pulse Anodic Stripping Voltammetry (DPASV) and Diffusive Gradients in Thin films (DGT). Due to root-induced alkalisation, pH reached about 7.3 in the rhizosphere whatever the initial bulk soil pH. Compared to the most acidic bulk soil (pH ≈ 4.8), Cu^{2+} activity decreased by three orders of magnitude in the rhizosphere while DPASV-Cu concentration decreased by 6-fold. DOM became the key driver of Cu dynamic speciation in the rhizosphere, where roots induced up to an order of magnitude increase in DOM concentration compared to bulk soils. This resulted in an increase in labile-Cu (both DPASV and DGT) concentrations, in spite of a decrease in Cu^{2+} activity. Model VI calculations supported a decrease in DOM binding capacity towards Cu in the rhizosphere. DPASV measurements unequivocally demonstrated that the increase in Cu lability in the rhizosphere solution can be attributed to a greater lability of organically-bound Cu. Collectively, our data introduce a consistent picture of root-induced changes of Cu dynamic speciation in the rhizosphere that were notably related to substantial alterations of DOM binding capacity.
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1. INTRODUCTION

According to the international organization for standardization, the environmental bioavailability of soil contaminants was recently defined at a conceptual level as the fraction of available contaminant in soil acquired by a target-organism through physiologically driven processes (Harmsen, 2007; ISO, 2008). Consequently, the characterisation of metal availability in soils and more particularly at the soil–root interface, i.e. in the rhizosphere, appears as a crucial step for determining metal bioavailability to plants.

Metal availability in soils depends both on the presence of a wide range of sorbents in the solid-phase such as organic matters (SPOM) and oxides as well as in the solution such as dissolved organic matters (DOM) and on physical–chemical

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parameters such as pH and ionic strength (Sauvé et al., 2000; Weng et al., 2002). Solid–solution partitioning of metals and free metal ion activity in soil solution are mainly controlled by soil pH as well as by DOM binding properties and concentration (Vulkan et al., 2000; Weng et al., 2002). Among metals, copper (Cu) exhibits a strong affinity for DOM. In the soil solution, total Cu concentration is usually positively correlated with DOM concentration (Zhou and Wong, 2001; Zhao et al., 2007b), while Cu^{2+} activity would tend to decrease at a given pH as DOM concentration increased (Minnich and McBride, 1987).

Plants are recognised to acquire metals in soil solution mainly as free ionic species. The amount of free metal ion in solution at the root surface is however several orders of magnitude lower than the amount taken up by plants, which involves an on-going re-supply of free metal ion from labile pools in soil (Kalis et al., 2008; Bravin et al., 2009b). An increasing amount of literature suggests that this re-supply could be often kinetically constrained either by metal diffusion in soil solution and/or by metal lability, i.e. the fraction of inorganic and organic metal complexes that contributes to the re-supply of depleted free metal in solution (Degryse et al., 2006a,b). The kinetic dimension of metal speciation, i.e. "dynamic speciation", shall thus be further accounted for when assessing metal bioavailability to plants (van Leeuwen et al., 2005). Potential changes in metal lability should have a substantial effect on dynamic metal speciation. However, to our knowledge, very few studies addressed metal lability in soil solution, and even less so in the rhizosphere. Using differential pulse anodic stripping voltammetry (DPASV) and binding resin measurements, Jeffery and Uren (1983) noted that most of Zn in solution was labile and Zn concentration was strongly related to pH, whereas most of Cu in solution was either moderately or non-labile and Cu concentration was altered both by pH and DOM. The concept of lability was further extrapolated to describe the labile pool of metals in the soil solid-phase. A range of techniques such as the diffusive gradients in thin films (DGT) or stable isotope fractionation was consequently developed to estimate metal lability in soil and was applied to the assessment of metal bioavailability to plants (Zhang et al., 2001; Oliver et al., 2006). Mimicking metal depletion that presumably occurs in the rhizosphere due to root uptake, DGT-measured metal concentration in soil was successfully correlated with metal concentration in a range of plant species (Degryse et al., 2009).

However, there is increasing evidence that additional root-induced chemical changes of notably pH and DOM can dramatically alter dynamic metal speciation in the rhizosphere. For example, we recently showed that Cu depletion in the rhizosphere of durum wheat (Triticum turgidum durum L.) grown in a strongly acidic soil was almost entirely driven by the root-induced increase in pH (i.e. rhizosphere alkalisation) while the contribution of root uptake had little impact (Bravin et al., 2009b). In addition, rhizodeposition (including root exudation) and the consequent increase in microbial activity usually contribute to an increase in DOM concentration in the rhizosphere which is correlated to a concomitant increase in metal concentration in solution (Cattani et al., 2006; Kim et al., 2010). Several authors fur-

ther observed a modification of DOM composition in the rhizosphere such as an increase in the concentration of low-molecular-weight organic acids and aromatic compounds (Degryse et al., 2008; Dessureault-Rompré et al., 2010; Kim et al., 2010). This suggests a substantial modification of DOM binding capacity towards metals in the rhizosphere, but direct experimental evidences are still lacking.

In a previous paper, we showed that durum wheat roots was able to induce a drastic increase in pH and DOM concentration in the rhizosphere when plants were exposed to a Cu-contaminated soil over a wide range of pH (Bravin et al., 2009a). Such a substantial rhizosphere alkalisation in a strongly acidic soil enabled us to understand why Cu bioavailability was fairly low and even comparable to Cu bioavailability measured in alkaline soil. However the impact of DOM on Cu dynamic speciation in the rhizosphere was not evaluated in details. Based on the same experiment and combining a range of analytical techniques and modelling, the present manuscript focuses on the alteration of Cu dynamic speciation in the rhizosphere as mediated by root-induced changes in pH and DOM.

2. MATERIAL AND METHODS

2.1. Soil properties and Ca(OH)$_2$ addition

The selected soil was sampled in the topsoil (0–20 cm) of a former vineyard of Southern France which received for decades repeated applications of Cu-based fungicides. Once sampled, the soil was air-dried, sieved at 2 mm and then analysed by a routine soil-testing laboratory (INRA-LAS, France) according to French (Agence française de normalisation, AFNOR, 1999) and international (ISO, 1999) procedures. The soil was strongly acidic ($pH_{CaCl2} = 4.1$) and moderately Cu-contaminated (total Cu = 184 mg kg^{-1}). Additional soil properties can be found in Bravin et al. (2009a).

The soil was limed with eight different rates of Ca(OH)$_2$ in order to obtain a wide range of pH values (Table 1). Prior to liming, soil samples were incubated for 13 days

Table 1
pH and dissolved organic carbon (DOC) concentration in the extracted soil solution of the large set ($n = 80$) of bulk soil and rhizosphere samples for the different rates of Ca(OH)$_2$ addition. Stars stand for a significant difference in pH ($P \leqslant 0.01$) and DOC concentration ($P \leqslant 0.05$) between bulk soil and rhizosphere for a given treatment.

	Ca(OH)$_2$ addition (µg g^{-1}) dry soil	pH		DOC (mg C dm^{-3})	
		Bulk soil	Rhizosphere	Bulk soil	Rhizosphere
T_0	0	4.8 ± 0.1	7.5 ± 0.1**	3.1 ± 0.8	35 ± 16*
T_1	184	5.3 ± 0.3	7.4 ± 0.1**	7 ± 2	26 ± 14*
T_2	323	5.6 ± 0.2	7.3 ± 0.1**	5 ± 1	41 ± 12*
T_3	439	5.9 ± 0.1	7.4 ± 0.1**	3.8 ± 0.6	42 ± 17*
T_4	811	6.4 ± 0.2	7.3 ± 0.2**	5 ± 2	25 ± 17*
T_5	1299	6.9 ± 0.2	7.3 ± 0.1**	8 ± 3	11 ± 5
T_6	1904	6.9 ± 0.1	7.3 ± 0.2**	4.2 ± 0.7	27 ± 12*
T_7	2626	7.5 ± 0.1	7.3 ± 0.1	5 ± 2	18 ± 7*

with a nutrient solution (see below in the soil stage of the RHIZOtest for the complete composition) at 70% water-holding capacity (20% mass water content). Eight rates of $Ca(OH)_2$ from 0 to 2626 $\mu g\,g^{-1}$ dry soil were applied to the incubated soil samples (Bravin et al., 2009a). In order to apply the same amount of calcium (Ca) whatever the liming treatment, a $CaCl_2$ solution of varying concentration was used so as to reach an even application rate of Ca of 35.4 $\mu mol\,g^{-1}$ in all soil samples. The water content was consequently raised to 100% water-holding capacity. Once treated with $Ca(OH)_2$ and/or $CaCl_2$, the soil samples were further incubated for another 3 days in order to equilibrate before starting the soil stage in the RHIZOtest.

2.2. Plant growth in RHIZOtest for sampling the rhizosphere

Plants were grown in a plant-based biotest, the RHIZOtest, which separates plant roots from soil by a 30-μm polyamide mesh to facilitate the collection of roots and rhizosphere. The RHIZOtest procedure was described in details by Chaignon and Hinsinger (2003) and Bravin et al. (2010).

Briefly, two seeds of durum wheat (cv. Acalou) per pot were germinated in $CaCl_2$ 600 μM and H_3BO_3 2 μM for 1 week, then grown in hydroponics for another 2 weeks in a complete nutrient solution of following composition (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 growth chamber parameters were set at (day/night): 25/20 °C, 75/70% of relative humidity and 450 μmol photons $m^{-2}\,s^{-1}$ of photon flux density.

For the soil stage, three-week-old seedlings with a dense, planar root mat were transferred onto a thin layer of pre-incubated soil (1.5-mm thick, 3 g dry soil) of each liming treatment. Root mat was continuously separated from the soil layer via a polyamide mesh. Soil layers were connected to 1-dm^3 of nutrient solution with a filter paper wick. The composition of this nutrient solution, also used for pre-incubating the soils, was the same as that used in hydroponics, except for KH_2PO_4 that was reduced to 50 μM and $CuCl_2$ and $NaFe(III)EDTA$, which were omitted to avoid substantial alteration of the concentration and speciation of soil solution Cu.

After 8 days of contact, the soil was considered as rhizosphere (Chaignon and Hinsinger, 2003). The root-induced chemical changes in the rhizosphere were measured relative to control soils which had been incubated in similar devices without plant (thereafter called bulk soil). Devices with and without plants were replicated five times for each liming treatment.

2.3. Soil solution extraction and analyses

After the 8-day contact period between plant roots and soil, the soil solution was considered to be in partial-equilibrium with the composition of the nutrient solution. Consequently, immediately after plant harvest, a solution of identical composition as the nutrient solution was used to extract the soil solution of each replicate (thereafter called the "large set", $n = 80$) as follows: 15 cm^3 of the nutrient solution were added to 1.92 g fresh soil (to yield a soil:solution ratio of 1:10 on a dry soil mass basis) in an end-over-end shaker for 2 h, and then centrifuged at 10,000 g for 10 min before filtration (0.45 μm, acetate cellulose filter). The remaining soil of the five replicates of each treatment for bulk soil and rhizosphere was then bulked, mixed homogeneously to obtain a composite sample per treatment (thereafter called the "small set", $n = 16$). Sample bulking was necessary to obtain enough soil and soil solution for subsequent Cu lability measurements using DGT and DPASV. Soil solution was extracted on the small set of composite samples by mixing 3.84 g fresh soil (i.e. 3 g dry soil equivalent) with 30 cm^3 of the nutrient solution as described above.

Within the day of extraction, Cu^{2+} activity ($p\{Cu^{2+}\} = -\log_{10}\{Cu^{2+}\}$) was measured in each soil solution of the small and large sets of samples with a Cu-ISE (DX 264, Mettler Toledo, USA) and a double-junction reference electrode (Inlab, Mettler Toledo). ISE measurements were calibrated as described by Rachou et al. (2007) and Bravin et al. (2009b). Free Cu ion concentration was calculated from $p\{Cu^{2+}\}$ and the activity coefficient. The latter was calculated with the extended Debye–Hückel equation (Ritsema, 1993). Immediately after $p\{Cu^{2+}\}$ determination, the soil solution pH was measured with a combined glass-electrode (6.0234.110, Metrohm, Switzerland). Sodium azide (NaN_3) 10^{-4} M was added to the small set of composite samples to stop any microbial activity, then the small and large sets of samples were stored at 4 °C. Total Cu and dissolved organic carbon (DOC) concentrations were measured (within a week) by graphite furnace atomic absorption spectrometry (GF-AAS, Varian Spectra AA-600 GTA-110, USA) and total organic carbon analyser (TOC-5000, Shimadzu, Japan), respectively. Free Cu ion concentrations appeared initially slightly larger than total Cu concentrations at pH < 5. ISE measurements on the small set of composite samples plus four additional samples ($n = 20$) were compared with $p\{Cu^{2+}\}$ estimated by DPASV measurements (see Section 2.5) and thus corrected accordingly (Fig. EA-1 in electronic annex). Free Cu concentration in all the samples of the large set was corrected similarly.

2.4. Modelling Cu^{2+} activity with Model VI

Free Cu ion activity in soil solution was also predicted in each bulk soil and rhizosphere extract of the large set of samples ($n = 79$, as one bulk soil replicate was lost during DOC measurement) by speciation modelling using the Humic Ion-Binding Model VI, thereafter called Model VI (Tipping 1998), which is included in the Windermere Humic Aqueous Model 6 (WHAM version 6.0.1). This modelling step aimed at estimating changes in DOM binding capacity towards Cu between the rhizosphere and the bulk soil. The input parameters were the concentration of major cations (Ca, Fe, K and Mg) and anions (NO_3 and SO_4), the concentration of Cu and fulvic acid, the free ion activity of Al and Fe and pH. Free ion activity of Al and Fe was considered to

be controlled by equilibrium with $Al(OH)_3$ and $Fe(OH)_3$, respectively, and were consequently calculated according to Tipping et al. (2003) with the following solubility products: $K_{s-Al,25} = 8.5$ and $K_{s-Fe,25} = 2.7$. Partial pressure of CO_2 was assumed to be that of the ambient atmosphere ($10^{-3.5}$ atm) and temperature was fixed at 25 °C.

According to a preliminary experiment performed on 25 soil samples including that used in the present study (Comment EA-1; Tables EA-1 and EA-2; Fig. EA-2 in electronic annex), the concentration of major cations and anions was fixed as follows (mM): Ca 2.4, K 1.5, Mg 0.88, NO_3 6 and SO_4 1. Total Cu and DOC concentration as well as pH were measured as detailed above. DOM concentration was calculated from DOC assuming that DOM contained 50% C. Humic substances are usually considered as the main pool of DOM driving its metal binding properties, whereas fulvic acid concentration in soil solution usually far exceeds that of humic acids (Bolan et al. 2011). Consequently, we considered that the fraction of DOM that effectively binds protons and metals behaved as a fulvic acid with the "generic" binding properties given in the corresponding data base of Model VI (Tipping 1998). Model VI calculations were thus fitted to ISE measurements independently for bulk soil and rhizosphere samples (see Section 3.3 for further details) by minimising the root mean square residual (RMSR):

$$RMSR = \left[\sum_{i=1}^{n}(p\{Cu^{2+}\}_{i,measured} - (p\{Cu^{2+}\}_{i,calculated})^2/n\right]^{1/2} \tag{1}$$

where n is the number of data points.

2.5. DPASV-Cu measurements in soil solution

Voltammetric measurements were performed on the soil solutions extracted from the small set of composite samples to assess the concentration of the most available forms of Cu and the lability of Cu complexes, especially that of organically bound-Cu, in the soil solution. The experimental procedure is briefly addressed below, while details can be found in electronic annex (see Comment EA-2). All voltammetric measurements were performed using a µAutolab potentiostat (EcoChemie, The Netherlands) equipped with a Metrohm 663 VA stand (Metrohm, Switzerland).

Pseudopolarographic measurements were initially performed on bulk soil and rhizosphere solutions of treatments T_0 (without lime addition) and T_7 (with the maximum rate of lime addition) by using a deposition time of 30 s at a selected range (from −1 or −0.85 to +0.1 V) of deposition potentials (E_{dep}) (Louis et al., 2008). Analysis of voltammetric peaks and construction of pseudopolarograms were manually performed using the Microsoft Excel software. Soil solutions of T_0 and T_7 samples were analysed by pseudopolarography first at initial pH and second after acidifying down to pH 2 with concentrated HNO_3 (suprapure grade, Merck). This acidification step enabled to fully dissociate organic Cu complexes and to determine the adequate E_{dep} (−0.1 V) enabling to measure only Cu^{2+} plus inorganically bound-Cu in non-acidified samples, defined in this study as "inorganic Cu" fraction. The additional fraction of Cu possibly measured at the most negative deposition potential thus corresponds to the reduction of organic Cu complexes, electrochemically dissociable, either due to weak stability constants or fast association rates (Nicolau et al., 2008; Feldmann et al., 2009). Both inorganic and organic fraction were defined as "DPASV" fraction, with respect to the one determined by DGT.

Bulk soil and rhizosphere solutions of the other treatments ($T_1–T_6$) were then analysed by voltammetry at two E_{dep} only, i.e. −0.85 and −0.1 V, before and after acidifying to pH 2. Peak areas were converted into Cu concentrations by calculating the method sensitivity using the voltammograms at pH 2. Free Cu ion activities and concentrations were calculated from the concentration of inorganic Cu measured at E_{dep} −0.1 V using the aqueous speciation model PHREEQC (version 2.14.3.2411) and the MINTEQ database of thermodynamic constants (Parkhurst and Appelo, 1999).

2.6. DGT-Cu measurements in soil

The DGT technique was performed on the remaining part of the small set of composite fresh soil samples to assess the potential mobilisation of Cu from the soil solution and presumably from the solid-phase. This fraction was defined as "DGT" fraction, with respect to the one determined by DPASV. Standard cylindrical DGT (Zhang et al., 2001) units with an active surface area of 3.14 cm² and a diffusive gel thickness of 0.083 cm were covered with 3.84 g fresh soil (i.e. 3 g dry soil equivalent) for 24 h at 25 °C. The mass of soil exposed to the DGT units was calculated to induce a depletion of less than 0.5% of total soil Cu. The resin was then retrieved and eluted in 1.2 cm³ of 1 M HNO_3 for 24 h. The concentration of eluted Cu was determined by GF-AAS. DGT measurements were recorded as the time-averaged concentration of Cu in the solution at the DGT–soil interface (Cu_{DGT}, mol dm⁻³), which was calculated according to:

$$m = Cu_{elution}(V_{HNO3} + V_{gel})/f_e \tag{2}$$

and:

$$Cu_{DGT} = m\Delta g/(M_{Cu}DAt) \tag{3}$$

where m is the mass of Cu (g) accumulated in the resin, $Cu_{elution}$ the concentration of Cu (g dm⁻³) in the eluted solution, V_{HNO3} the volume (dm³) of HNO_3 used for elution, V_{gel} the volume ($1.5 \cdot 10^{-4}$ dm³) of solution in the gel, f_e the elution factor assigned to 0.8 (Zhang and Davison, 1995), Δg the thickness ($9.3 \cdot 10^{-3}$ dm) of the diffusive layer (diffusive gel plus filter paper), M_{Cu} the molar mass (63.5 g mol⁻¹) of Cu, D the diffusion coefficient ($6.23 \cdot 10^{-8}$ dm² s⁻¹) in the diffusive layer, A the active surface area (dm²) of the DGT unit and, t the soil-DGT device contact time (s).

2.7. Statistics

Analyses of variance (HSD Tuckey's test) as well as simple and multi-linear regressions were performed with Statis-

tica (version 6, StatSoft). The level of statistical significance was represented by *, ** and *** for $P \leqslant 0.05$, $P \leqslant 0.01$ and $P \leqslant 0.001$, respectively.

3. RESULTS

3.1. Root-induced alkalisation and increase in DOC concentration in the rhizosphere solution

As a consequence of $Ca(OH)_2$ addition, bulk soil pH increased from 4.6 to 7.6 (Table 1). Whatever the initial soil pH, rhizosphere pH ranged between 6.9 and 7.6 and was significantly higher ($P \leqslant 0.01$) than bulk soil pH in all treatments but T_7 (Table 1; Bravin et al., 2009a). Whatever the bulk soil pH, DOC concentration was larger ($P \leqslant 0.05$) in the rhizosphere than in the corresponding bulk soil, except for T_5 where the difference was not significant (Table 1; Bravin et al., 2009a). Similar results were found for pH and DOC as measured in the small set of composite soil samples (Table EA-3 in electronic annex).
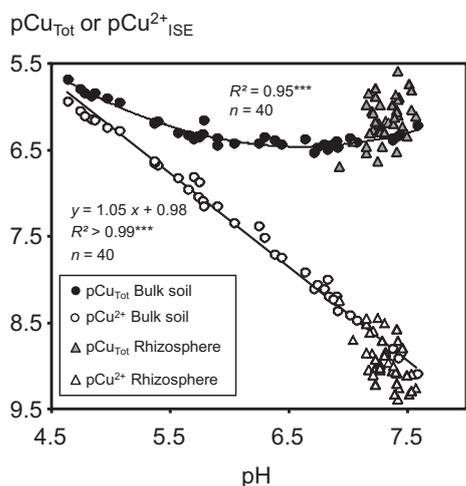
Fig. 1. Total Cu (pCu) and Cu^{2+} (pCu^{2+}) concentrations in bulk soil and rhizosphere solutions as a function of pH for the large set of samples.

3.2. Measured concentrations of total Cu and Cu^{2+} in solution

In the bulk soil, we found that both total Cu and Cu^{2+} concentrations in soil solution, respectively expressed as pCu_{Tot} and pCu^{2+} (i.e. $-\log_{10}[Cu]$ and $-\log_{10}[Cu^{2+}]$), were strongly correlated with pH (Fig. 1). The results obtained for the small set of composite samples (Fig. EA-3 in electronic annex) were in line with those obtained for the large set (Fig. 1). Free Cu ion concentration decreased linearly with increasing bulk soil pH, ranging from pCu^{2+} 5.1 to 9.1 (Fig. 1).

In the rhizosphere, total Cu concentration ranged from pCu 5.6 to 6.7. Total Cu concentration was not significantly correlated with soil solution pH but linearly and positively correlated with DOC concentration in the rhizosphere ($R^2 = 0.86^{***}$, $n = 40$; Fig. 2a). In contrast with bulk soil, Cu^{2+} concentration in the rhizosphere soil solution remained rather low, with pCu^{2+} ranging from 8.2 to 9.5. Free Cu ion concentration was mainly correlated with DOC concentration ($R^2 = 0.61^{***}$, $n = 40$; Fig. 2b) and poorly but significantly correlated with pH ($R^2 = 0.39^{***}$, $n = 40$), ultimately yielding the following multi-linear regression:

$$p[Cu^{2+}] = 0.59(\pm 0.10)\log_{10}[DOC] + 0.7(\pm 0.2)pH + 3(\pm 2)$$
(4)

$$R^2 = 0.70^{***} \quad n = 40$$

It is noteworthy that, contrary to total Cu, Cu^{2+} concentration decreased with increasing DOC concentration. In the small set of composite soil samples, a linear relationship between pCu and DOC concentration was found as in the large set (Fig. EA-4a in electronic annex), while pCu^{2+} was not so clearly related to DOC concentration (Fig. EA-4b in electronic annex).

3.3. Modelling Cu^{2+} activity in solution

DOM was described as a single pool in bulk soil. DOM concentration in the rhizosphere was larger than the mean DOM concentration in the five bulk soil replicates of the
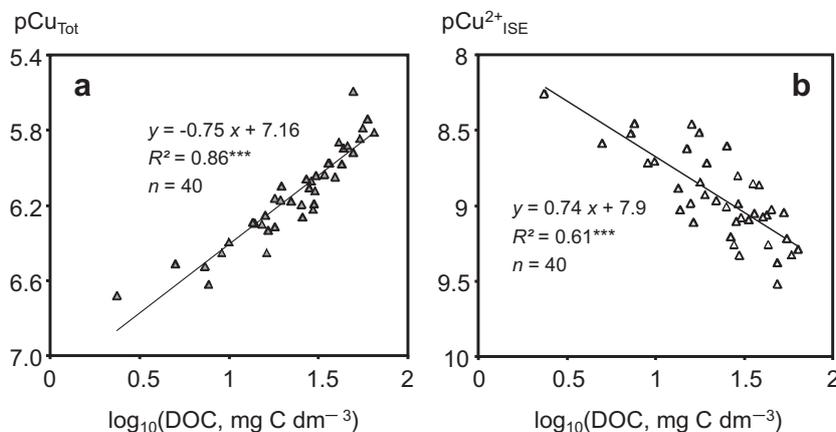

Fig. 2. Total Cu (pCu) (a) and Cu^{2+} (pCu^{2+}) (b) concentrations in rhizosphere solutions as a function of dissolved organic carbon (DOC) concentration for the large set of samples.

corresponding treatment for 37 out of 40 rhizosphere samples (see Table EA-4 in electronic annex). Consequently, DOM in the rhizosphere was subdivided into two pools of distinct Cu binding capacity. We considered that the first pool of DOM in the rhizosphere was inherited from bulk soil and we thus fixed its concentration for each replicate at the mean concentration of DOM measured in the five bulk soil replicates of the corresponding treatment. The second pool of DOM was considered to be inherited from root activities and root-induced microbial activities in the rhizosphere and was calculated as the difference between total DOM concentration in the rhizosphere and DOM concentration in the first pool (Table EA-4 in electronic annex).

Model VI calculations of Cu^{2+} activity were primarily fitted to ISE measurements for the bulk soil samples ($n = 39$) by adjusting fulvic acid concentration as a percent-

age of DOM concentration, the remaining DOM being considered as inert. The same percentage of DOM behaving as fulvic acid was applied to the first pool of DOM in the rhizosphere (i.e. pool inherited from bulk soil). Model VI calculations of Cu^{2+} activity were then fitted to ISE measurements for the rhizosphere samples ($n = 40$) by adjusting fulvic acid concentration as a percentage of DOM concentration in the second pool. Free Cu ion activities in bulk soil and rhizosphere solutions were calculated using the input data shown in electronic annex (Table EA-5 in electronic annex).

In bulk soil, we were able to fit fairly well the measured Cu^{2+} activities with Model VI ($RMSR = 0.32$ p$\{Cu^{2+}\}$ unit) when considering that 42% of DOM behaved as a fulvic acid (Fig. 3a and b). When DOM reactivity was considered to be similar in the rhizosphere (i.e. a single pool of DOM in which 42% behaved as a fulvic acid), Cu^{2+} activities calculated with Model VI substantially under-estimated ($RMSR = 0.70$ p$\{Cu^{2+}\}$ unit) Cu^{2+} activity measurements (Fig. 3a).

Alternatively, DOM reactivity in the rhizosphere can be described as two distinct pools of DOM, a first pool inherited from bulk soil in which 42% of DOM behaved as a fulvic acid and a second pool inherited from root-induced activities in which 27% of DOM behaved as a fulvic acid.
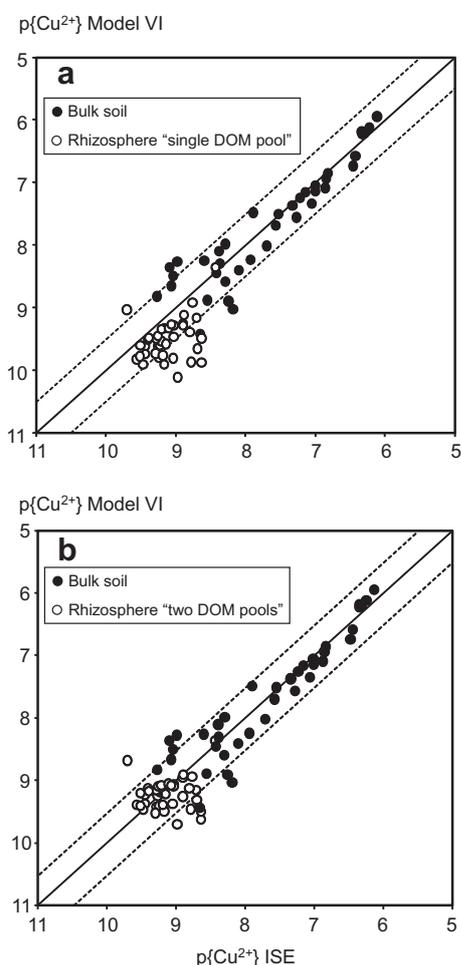
Fig. 3. Comparison between Cu^{2+} activity calculated with Model VI (p$\{Cu^{2+}\}$ Model VI) and Cu^{2+} activity measured with an ion-selective electrode (p$\{Cu^{2+}\}$ ISE) in bulk soil and rhizosphere solutions. Free Cu ion activity was calculated by considering DOM in the rhizosphere either (a) as a single pool with a similar binding capacity than that in bulk soil or (b) as two pools of distinct binding capacity (see Section 3.3 for further details). Solid lines stand for the 1:1 line. Dashed lines stand for 1:1 line ± 0.5 p$\{Cu^{2+}\}$ unit.
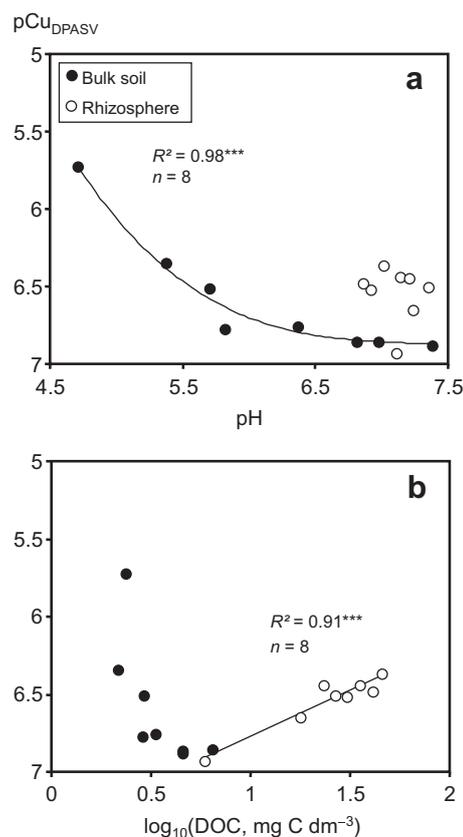
Fig. 4. Concentration of labile Cu as measured by DPASV (pCu$_{DPASV}$, $E_{dep} = -0.85$ V) in bulk soil and rhizosphere solutions as a function of pH (a) or dissolved organic carbon (DOC) concentration (b).

According to these assumptions, Cu^{2+} activities calculated with Model VI fitted fairly well ($RMSR = 0.37$ p$\{Cu^{2+}\}$ unit) with the measured Cu^{2+} activities in the rhizosphere (Fig. 3b).

DPASV-Cu lability, % total Cu in solution
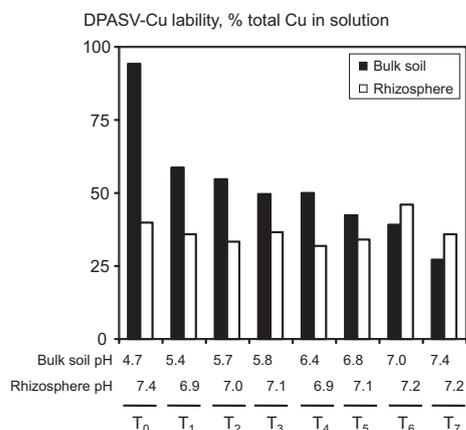
Fig. 5. Copper lability measured by DPASV ($E_{dep} = -0.85$ V) in soil solution as a percentage of total Cu in bulk soil and rhizosphere solutions for the different rates of Ca(OH)$_2$ addition.

3.4. Labile Cu in solution as measured by DPASV

Voltammetric (DPASV) measurements of labile Cu in solution were performed on the small set of composite soil samples only ($n = 16$), given the rather large volume of solution which is necessary for this technique. DPASV-Cu concentration as measured at E_{dep} -0.85 V showed clearly distinct relationships with pH and DOC concentration in bulk soil and rhizosphere solutions (Fig. 4), with similar patterns than those observed for total Cu concentration in solution. In bulk soil solutions, DPASV-Cu concentration decreased sharply and almost linearly as pH increased up to pH 5.8 and then remained fairly stable at about pCu 6.8 (Fig. 4a). In the rhizosphere solutions, DPASV-Cu concentration was independent of soil solution pH and was linearly, positively correlated with DOC concentration ($R^2 = 0.91$[***], $n = 8$; Fig. 4b). DPASV-Cu concentration was up to 2.5-fold larger in the rhizosphere than in the corresponding bulk soil at bulk soil pH higher than ca. 5.5, except in the treatment T_5 which exhibited a particularly low DOC concentration in the rhizosphere (Fig. EA-5 in electronic annex). DPASV-Cu lability, i.e. DPASV to total solution Cu concentration ratio, differed also substantially between bulk soil and rhizosphere (Fig. 5). In bulk soil, DPASV-Cu lability decreased from 94 to 27% with increas-
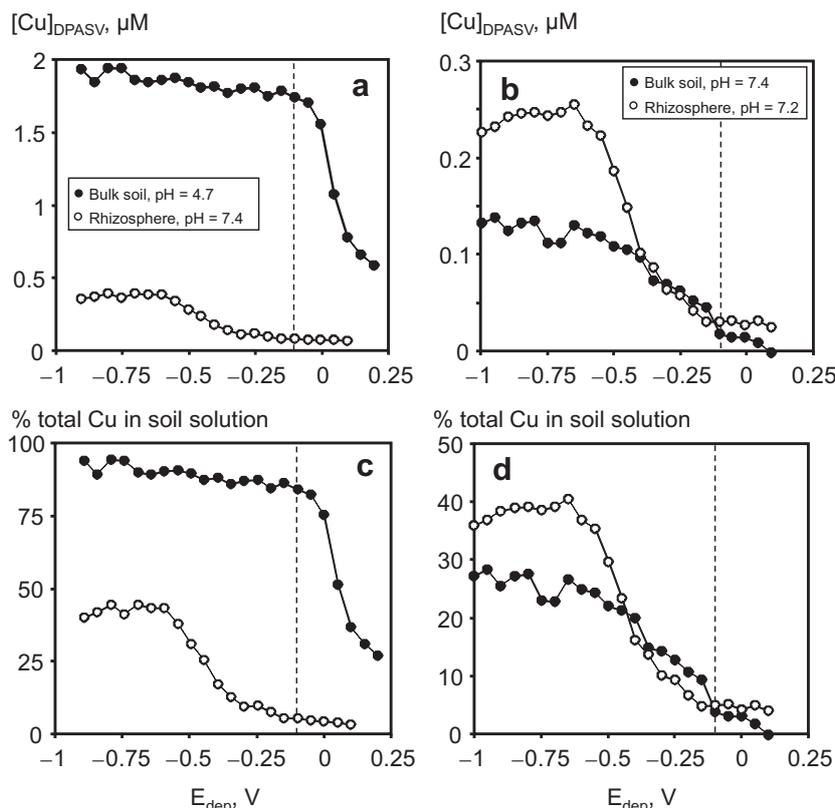
Fig. 6. Pseudopolarograms showing Cu concentration ([Cu]$_{DPASV}$) and the percentage of total Cu in solution occurring as labile Cu as measured by DPASV in bulk soil and rhizosphere solutions as a function of the DPASV deposition potential (E_{dep}). Measurements were performed for soils treated without Ca(OH)$_2$ addition (T_0) (a and c) and with the maximum rate of Ca(OH)$_2$ addition (T_7) (b and d). Vertical dashed lines at $E_{dep} = -0.1$ V stand for the threshold between labile Cu occurring as organic complexes (left side) and labile Cu occurring as inorganic Cu (free ion plus inorganic complexes; right side) (see Section 2.5 for further details).
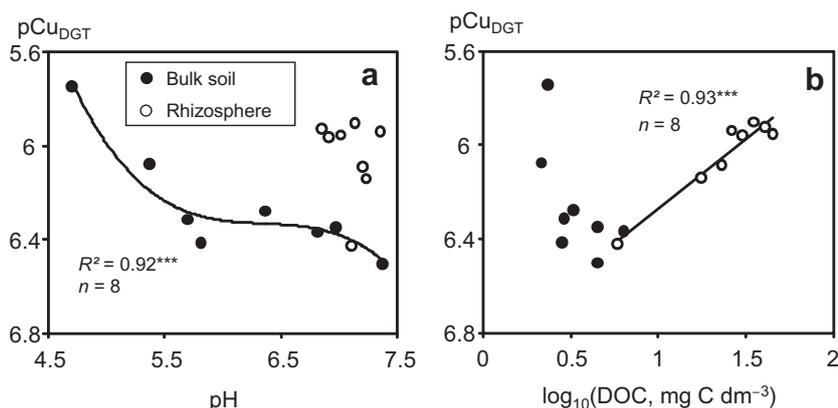
Fig. 7. DGT-Cu concentration (pCu_{DGT}) in bulk soil and rhizosphere solutions as a function of soil solution pH (a) or dissolved organic carbon (DOC) concentration (b).

ing pH. In the rhizosphere, DPASV-Cu lability remained fairly stable between 32% and 46%. Consequently, DPASV-Cu lability was larger in the rhizosphere than in the bulk soil from neutral pH (Fig. 5).

Pseudopolarographic measurements were performed on bulk soil and rhizosphere solutions of the two extreme liming treatments T_0 and T_7. In the treatment T_0 where a considerable alkalisation occurred in the rhizosphere, both DPASV-Cu concentration and lability were lower in the rhizosphere than in the bulk soil whatever the deposition potential (Fig. 6a,c). In the treatment T_7 where pH did not significantly change in the rhizosphere and where DOC concentration considerably increased in the rhizosphere (Table 1; Table EA-3 in electronic annex), pseudopolarograms of bulk soil and rhizosphere solutions were fairly similar at E_{dep} higher than -0.45 V (Fig. 6b,d). However below -0.45 V, both DPASV-Cu concentration and lability were up to 2.2-fold larger in the rhizosphere than in the bulk soil.
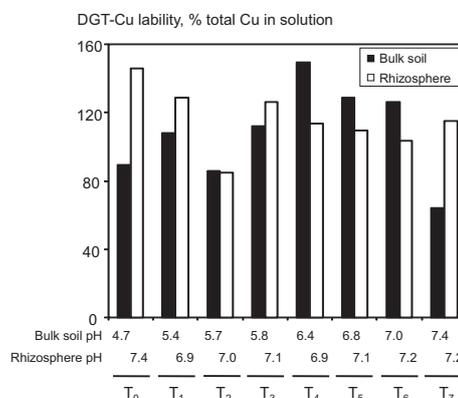
Fig. 9. Copper lability measured by DGT in soil as a percentage of total Cu in bulk soil and rhizosphere solutions for the different rates of $Ca(OH)_2$ addition.

3.5. Labile Cu in soil as measured by DGT

DGT measurements of labile Cu in soil were performed on the small set of composite soil samples only ($n = 16$), given the rather large amount of soil which is necessary to apply this technique. The time-averaged concentration of Cu in the solution at the DGT–soil interface, i.e. Cu_{DGT}, showed clearly distinct relationships with pH and DOC concentration in bulk soil and rhizosphere solutions (Fig. 7), with similar patterns to those observed for total- and DPASV-Cu concentrations in solution. In bulk soils, Cu_{DGT} decreased almost linearly as pH increased up to pH 5.7 and then remained fairly stable at pCu_{DGT} 6.4 (Fig. 7a). In contrast, Cu_{DGT} was independent of pH in the rhizosphere and was linearly, positively correlated with DOC concentration in the rhizosphere (Fig. 7b). Cu_{DGT} was up to 3.3-fold larger in the rhizosphere than in the corresponding bulk soil at bulk soil pH higher than ca. 5, except in the treatment T_5 which exhibited a particularly low DOC concentration in the rhizosphere (Fig. EA-6 in electronic annex). DGT-Cu (as pCu_{DGT}) and DPASV-Cu (as pCu_{DPASV}) concentrations were linearly correlated both in the bulk soil and the rhizosphere (Fig. 8). DPASV-Cu

Fig. 8. DGT-Cu concentration (pCu_{DGT}) in bulk soil and rhizosphere solutions as a function of DPASV-Cu concentration (pCu_{DPASV}). Dashed lines stand for the 1:1 line.

concentration was lower than Cu_{DGT} both in the bulk soil and the rhizosphere, except for the most acidic bulk soil (pH = 4.7) where Cu in solution was almost fully DPASV-labile and thus DPASV-Cu concentration was equal to both Cu_{DGT} and total Cu concentration in solution.

DGT-Cu concentration (Cu_{DGT}) was roughly equal to total Cu concentration in both rhizosphere and bulk soil solutions. DGT-Cu lability, i.e. DGT to total solution Cu concentration ratio, was rather stable with an average value of $112 \pm 23\%$ and was related neither to pH nor to DOM concentration (Fig. 9).

4. DISCUSSION

Plant roots are able to induce considerable changes of chemical properties in the rhizosphere as recently reviewed by Hinsinger et al. (2009). In the present work with durum wheat we report on two major root-induced chemical processes, namely (i) an alkalisation of up to +2.8 pH units and (ii) an increase in DOM concentration (Table 1; Table EA-3 in electronic annex). Rhizosphere alkalisation can be considered as a plant strategy to alleviate aluminium and manganese toxicities as well as phosphorus deficiency which are likely to occur in strongly acidic soils (Kochian et al., 2004; Hinsinger et al., 2009), such as the 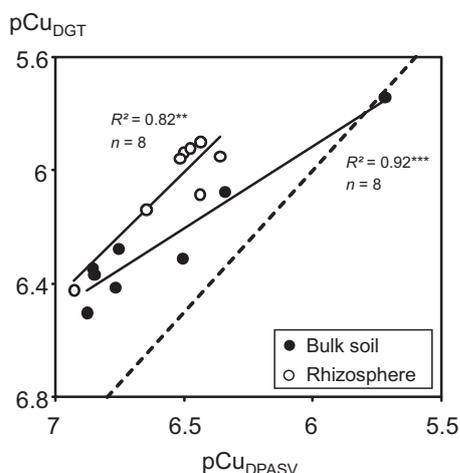unlimed soil (treatment T_0) used in our work. In addition, the increase in DOM concentration in the rhizosphere is usually associated both with rhizodeposition, especially root exudation, and with the subsequent enhancement of microbial activities in the rhizosphere which may ultimately lead to an increased turnover of soil organic matter (Khalid et al., 2007; Jones et al., 2009). The aim of the present study was thus to address how such root-induced alteration of pH and DOM can alter the dynamic speciation of Cu in the rhizosphere of durum wheat, with a specific focus on changes in DOM binding capacity towards Cu.

4.1. Copper dynamic speciation as affected by rhizosphere alkalisation

Overall, our results stressed the drastic effect of pH on Cu dynamic speciation in the bulk soil. In close agreement with the literature (Jeffery and Uren, 1983; McBride 1989), total Cu concentration in soil solution exhibited a U-shape when plotted against pH, with maximal concentrations occurring at pH values lower than 5.5. Neither concentration nor intrinsic binding capacity (as related to binding site density and thermodynamic binding constants) of DOM changed importantly over the wide range of investigated bulk soil pH (Table 1 and Fig. 3). The increasing ability of DOM to bind Cu with increasing pH is rather related to the deprotonation of DOM binding sites (van Riemsdijk et al., 2006). This suggests that the little dependence of total Cu concentration in soil solution on pH at pH values larger than 5.5 was mainly due to similar binding capacity of DOM and organic matters in the soil solid-phase (SPOM). Copper speciation in bulk soil solutions also agreed with the literature (McBride, 1989; Vulkan et al., 2000; Ma et al., 2006), as shown by the linear decrease in Cu^{2+} con-

centration as pH increased. Copper lability was also considerably altered by pH. In the unlimed bulk soil (T_0, pH ≈ 4.7), 94% of Cu in soil solution was DPASV labile (E_{dep} −0.9 V) and this percentage decreased with increasing pH, down to 27% in the most alkaline bulk soil solution (Figs. 4a and 5). Furthermore, the percentage of labile Cu in soil solution that comes from the dissociation of organically bound-Cu increased from 10% at the lowest pH value to about 90% when bulk soil pH was neutral to alkaline (results not shown). Jeffery and Uren (1983) described an identical pattern for labile Cu, as measured by DPASV and a chelex resin, as a function of pH in the soil solution of Cu-spiked soil. Alternatively, DGT-Cu lability in soil did not exhibit any pH dependency, as DGT-Cu concentration (i.e. Cu_{DGT}) was roughly equal to total Cu concentration in solution whatever the bulk soil pH. This means that the lability of Cu complexes in the solution and the solid-phase of bulk soils was high enough to sustain the uptake rate of Cu by DGT at the deployment-time scale used (i.e. 24 h).

In comparison with bulk soil, root-induced alkalisation, which resulted in a rhizosphere pH of about 7.3 ± 0.1 whatever the initial bulk soil pH, dramatically altered Cu dynamic speciation in the rhizosphere. The effect of alkalisation was most dramatic in the most acidic, unlimed bulk soil (T_0, pH ≈ 4.7) where Cu^{2+} concentration decreased by as much as three orders of magnitude and Cu lability in soil solution decreased more than 2-fold in the rhizosphere (Figs. 1 and 5). Rhizosphere alkalisation thus enabled plant roots to drastically decrease their exposure to Cu. This further supports the recent findings of Cornu et al. (2007), Bravin et al. (2009a) and Chaignon et al. (2009) who evidenced that some plant species (tomato, durum wheat and oilseed rape, respectively) were able to significantly alkalise their rhizosphere and thereby reduce Cu bioavailability to plants grown in strongly acidic, Cu-contaminated soils.

4.2. Copper dynamic speciation as affected by concentration and binding capacity of DOM in the rhizosphere

Root-induced alkalisation was such that the rhizosphere pH varied very little (7.3–7.5) compared with bulk soil (4.7–7.5). Copper dynamic speciation in the rhizosphere was therefore hardly related at all to rhizosphere pH. In contrast, the considerable quantitative (related to concentration) and qualitative (related to binding capacity) changes in DOM which occurred in the rhizosphere played a crucial role in determining Cu dynamic speciation in the rhizosphere.

The increase in DOM concentration in the rhizosphere was followed by a related increase in total Cu concentration in the soil solution (Fig. 2a). This led to total Cu concentration in some rhizosphere soil solutions reaching that of the most acidic bulk soil despite a considerable pH difference (Fig. 1). Such concomitant increases of DOM concentration and total Cu concentration in soil solution had been previously reported both in the presence and absence of plants (Jeffery and Uren, 1983; Zhao et al. 2007a,b; Kim et al., 2010). As for total Cu concentration in the rhizosphere solutions, DOM promoted an increase in DPASV- and DGT-

Cu concentrations (Figs. 4b and 7b). Cattani et al. (2006) similarly reported an increase in DGT-Cu concentration concomitantly to an increase in DOM concentration in the rhizosphere of maize grown in a calcareous, Cu-contaminated soil. Although DPASV and DGT exhibit rather different kinetic windows (i.e. a smaller kinetic window for DPASV, with mainly fast dissociating metal complexes involved, than for DGT with a larger contribution of slowly dissociating metal complexes), DPASV-Cu and DGT-Cu concentrations were similarly altered by DOM in the rhizosphere and consequently gave a convergent picture of the effect of DOM concentration on labile Cu concentration (Fig. 8). Free Cu ion concentration was also mainly controlled by DOM concentration in the rhizosphere solutions (Fig. 2b). However, opposite to total and labile Cu, Cu^{2+} concentration decreased with increasing DOM concentration. This increase in DOM concentration was thus able to shift the equilibrium between Cu bound to DOM, Cu bound to organic matters in the soil solid-phase and free Cu^{2+} in the soil solution, as previously suggested by Minnich and McBride (1987). An additional Model VI calculation considering the chemical conditions measured in the rhizosphere solutions (more particularly DOM concentrations, slightly alkaline pH), a single pool of DOM reacting as a fulvic acid and a single pool of SPOM reacting as a humic acid (42% of DOM and SPOM was considered as reactive) simulated a similar decrease in Cu^{2+} concentration as DOM concentration increases (results not showed). This suggests that the decrease in Cu^{2+} concentration as DOM concentration increases is not specific to the rhizosphere but it is likely a more generic mechanism that can be also observed in alkaline bulk soils provided that DOM concentration increases without any major change in the other chemical properties of soil solution.

Our results further suggest that the resulting dynamic speciation of Cu not only depends on the amount of DOM in the rhizosphere but also on the quality of DOM and notably on its intrinsic binding properties towards Cu. We did not characterise the nature of the organic compounds occurring in the soil solution, neither in the bulk soil nor in the rhizosphere. Instead we rather focused on the characterisation of DOM reactivity towards metals, i.e. by studying the intrinsic binding properties and the lability of Cu-DOM complexes. In bulk soil, Cu speciation was adequately modelled by considering that 42% of DOM behaved as a fulvic acid while the remainder was considered as inert (Fig. 3). This percentage of reactivity was lower than published values of 65% and 69% fixed to fit measurements of Cu^{2+} activity using Model VI and NICA-Donnan formalisms in Cu-contaminated soils covering a wide range of pH values (Vulkan et al., 2000; Weng et al., 2002), but it was in the range of 40–80% reported more globally for fulvic acid in surface waters and soil solutions (Tipping, 1998). In contrast with bulk soil, when a similar binding capacity was applied to DOM in the rhizosphere, Cu^{2+} activity was substantially underestimated with Model VI (Fig. 3a). Consequently, we further assumed that DOM in the rhizosphere could be split into two pools of distinct binding capacity, a first pool with reactivity similar to that of the bulk soil and a second pool with a specific reactivity inherited from either root activities

(rhizodeposition) and root-induced changes in microbial activities in the rhizosphere. According to this assumption, Cu speciation was adequately modelled by considering that only 27% of the second pool of DOM behaved as a fulvic acid while the remainder was considered as inert (Fig. 3b). Although it is likely oversimplified to describe DOM reactivity in the rhizosphere with two distinct pools, these results strongly suggest a lower binding capacity of DOM in the rhizosphere of durum wheat than in the bulk soil. Using Model VI, this lower binding capacity was interpreted as a lower density of binding sites on DOM (i.e. a lower amount of binding sites per quantity of DOM) than the generic fulvic acid used in Model VI. While this was not addressed in the present Model VI calculations, a lower affinity for Cu (i.e. lower thermodynamic binding constants) of DOM in the rhizosphere may also partly explain such a lower binding capacity. The respective role of the lower density of binding sites and the lower affinity for metals that both contribute to the lower binding capacity of DOM in the rhizosphere should deserve further investigations. These results also point out the need for further understanding DOM composition in the rhizosphere to ultimately refine model estimation of resulting binding properties. Roots and microorganisms usually exude a complex mixture of Cu-chelating compounds in the rhizosphere, from low molecular weight organic anions (carboxylates), amino-acids (e.g. phytosiderophores for graminaceous species as durum wheat) and phenolics to high molecular weight organic molecules such as mucilage (root and microbial polysaccharides which contain uronic acids) and proteins, that exhibit a wide range of metal binding capacity (Morel et al., 1986; Mench et al., 1987; Jones et al., 2009). It should also be stressed that in most plant species the bulk of root exudates are sugars and a range of other compounds (Jones et al., 2009) which would hardly complex any metal ion at all and would result in a decrease in the overall reactivity of rhizosphere DOM when expressed on a C mass basis. An alternative hypothesis that could explain the decrease in DOM binding capacity in the rhizosphere would involve the alteration of the conformational structure of the soluble humic substances in the rhizosphere relative to bulk soil. Organic anions, i.e. potential root exudates, have been reported to induce a disruption of humic substances in smaller molecules (Piccolo et al., 2003). In addition, Wang et al. (2010) recently reported a decrease in the log K_{Cu} value with decreasing molecular weight of DOM fractions originated from chicken manure, from log $K = 5.4$ in the >10 kDa fraction down to log $K = 2.7$ in the <1 kDa fraction. Consequently, such changes in the molecular size of dissolved humic substances in the rhizosphere could be responsible for the reduced binding capacity of DOM. Unfortunately, to our knowledge, the explicit relationship between conformational changes in humic molecules and their metal-binding affinity has not been addressed so far. Whereas additional studies are needed to assess the genericity of the lower binding capacity of DOM in the rhizosphere, this finding should be more consistently considered when geochemical models are used to calculate metal speciation in the rhizosphere of common crop species.

Beyond equilibrium considerations of DOM binding capacity, a more dynamic approach of Cu speciation in

soils was also addressed by measuring the lability of Cu-DOM complexes using DPASV. The pseudopolarograms performed on the bulk soil and the rhizosphere of the most alkaline soil (treatment T_7) demonstrated the major effect of DOM on DPASV-Cu lability in the soil solution (Fig. 6b,d). DPASV-Cu lability exhibited similar shapes in bulk soil and rhizosphere for E_{dep} larger than -0.45 V, which suggests that inorganic Cu complexes and the most readily dissociable organic Cu complexes have the same lability in the bulk soil and the rhizosphere. However, at lower E_{dep}, DPASV-Cu lability became significantly larger in the rhizosphere (Fig. 6d). To our knowledge, it is the first direct evidence of a larger lability of some organic Cu complexes in the rhizosphere solution, relative to that of the bulk soil solution. It physically means that compared with bulk soil a substantial part of organic Cu complexes in the rhizosphere solution exhibit high dissociation rate constant in absolute value or relatively to association rate constant (i.e. thermodynamic equilibrium constant is lower in the rhizosphere than in the bulk soil). It is noteworthy that this finding is consistent with the decrease in DOM binding capacity in the rhizosphere as suggested above from the calculation of Cu speciation using Model VI. The findings of Degryse et al. (2008) also support a larger lability of organically-bound Cu and Zn in the rhizosphere as related to root exudation of specific organic ligands by Zn-deficient plants. Conversely, Dessureault-Rompré et al. (2010) noted a decrease in the lability of Cd and Zn as measured by square wave ASV in the rhizosphere of *Thlaspi caerulescens* in comparison with bulk soil, which was attributed to the considerable sink effect induced by the roots of this hyperaccumulator plant species. The authors further suggested rhizosphere accumulation of metals bound with DOM exhibiting a higher aromaticity than DOM in the bulk soil, presumably supporting the decrease in metal lability with time. Concerning common crop species, which induce a much lower sink effect at the root surface than hyperaccumulating plant species, our data suggest that metal lability would rather increase in the rhizosphere. This increase in metal lability in the rhizosphere shall be a major driver of metal bioavailability to plants. In nutrient solutions, Degryse et al. (2006a,b) measured an increase in Cd, Cu and Zn uptake in plants when the lability of organic metal complexes was increasing. For field-grown plants, the combination of the decrease in Cu lability in strongly acidic soils as a consequence of root-induced alkalisation and the increase in Cu lability in the rhizosphere of alkaline soils also strongly supports the findings of Michaud et al. (2007) who did not observe any significant difference in Cu uptake in durum wheat cropped in soils exhibiting strongly acidic to alkaline pH.

5. CONCLUSION

This study is the first report of the application of complementary speciation techniques to elucidate metal dynamic speciation in soil and more particularly in the peculiar micro-environment of the rhizosphere. Collectively, our data introduce a consistent picture of root-induced changes in Cu dynamic speciation in the rhizosphere of durum wheat. Root-induced alkalisation considerably altered Cu dynamic speciation in the most acidic soils (pH < 5.5) by decreasing Cu^{2+} concentration and Cu lability. In the rhizosphere, soil pH influence almost disappeared as rhizosphere pH was neutral to slightly alkaline (7.3–7.5) whatever the initial bulk soil pH (4.8–7.5), and DOM thus became the major driver of Cu dynamic speciation. The increase in DOM concentration in the rhizosphere resulted in a decrease in Cu^{2+} concentration in solution but, in the mean time, in a mobilisation of Cu sorbed from the soil solid-phase and an increase in the concentration of labile Cu in soil. The percentage of reactive DOM (binding capacity towards Cu) as evaluated using Model VI decreased in the rhizosphere in comparison with bulk soil. Consistently, we also found an increase in the lability of organically bound-Cu in the rhizosphere solution compared to bulk soil. These results point to hypothetical links between the change in DOM binding capacity and DOM composition in the rhizosphere that need to be further investigated to enable an in-depth understanding of metal biogeochemistry in the rhizosphere and metal bioavailability to plants.

APPENDIX A. SUPPLEMENTARY DATA

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.gca.2012.01.031.

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