ORIGINAL ARTICLE

Copper uptake and phytotoxicity as assessed in situ for durum wheat (*Triticum turgidum durum* L.) cultivated in Cu-contaminated, former vineyard soils

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Abstract This work assessed in situ, copper (Cu) uptake and phytotoxicity for durum wheat (*Triticum turgidum durum* L.) cropped in a range of Cu-contaminated, former vineyard soils (pH 4.2–7.8 and total Cu concentration 32–1,030 mg Cu kg^{-1}) and identified the underlying soil chemical properties and related root-induced chemical changes in the rhizosphere. Copper concentrations in plants were significantly and positively correlated to soil Cu concentration (total and EDTA). In addition, Cu concentration in roots which was positively correlated to soil pH tended to be larger in calcareous soils than in non-calcareous soils. Symptoms of Cu phytotoxicity (interveinal chlorosis) were observed in some calcareous soils. Iron (Fe)–Cu antagonism was found in calcareous soils. Rhizosphere alkalisation in the most acidic soils was related to decreased CaCl$_2$-extractable Cu. Conversely, water-extractable Cu increased in the rhizosphere of both non-calcareous and calcareous soils. This work suggests that plant Cu uptake and risks of Cu phytotoxicity in situ might be greater in calcareous soils due to interaction with Fe nutrition. Larger water extractability of Cu in the rhizosphere might relate to greater Cu uptake in plants exhibiting Cu phytotoxic symptoms.

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Introduction

The protective effect of copper (Cu) salts against the mildew of grape vine has been discovered at the end of the nineteenth century. Since then, the repeated use of Cu salts as fungicides sprayed on vines has caused Cu contamination of most vineyard soils in various countries (Brun et al. 2001; Chaignon et al. 2003; Pietrzak and McPhail 2004; Cattani et al. 2006). In the Languedoc region (Southern France), more than a third of the former vineyard surface area has been replaced by other crops, mainly durum wheat (*Triticum turgidum durum* L.). While problems of Cu phytotoxicity have been seldom reported in vine, such problems have been found especially in cereals grown in calcareous soils in this region (Braun 2006) as well as in Switzerland (Coullery 1997). These findings were rather surprising as the bioavailability and phytotoxicity of metals is often thought to be minimal at higher pH.

In both soil solution and solid phase, Cu is mainly associated with organic matter by complexation or adsorption (Sauvé et al. 1997). Copper can also be adsorbed onto the surfaces of clays and Fe or Mn

oxides, co-precipitated with carbonates and phosphates or present in the lattice of primary silicate minerals (McBride 1981). The distribution of Cu among these various soil components can be defined as the solid phase speciation of soil Cu. The chemically mobile fraction of an element is defined as the sum of the amount in soil solution and the amount retained by the solid phase that can be easily transferred into soil solution (Rieuwerts et al. 1998). The solid phase speciation of Cu will greatly influence the chemical mobility and hence, the amount of Cu potentially taken up by plants. This will thus depend on those soil chemical properties that govern the solid phase speciation of Cu. Actually, at acidic pH, dissolved Cu will increase because of its weaker adsorption and so will the free cupric ion activity (Sauvé et al. 1997). In addition, with increasing pH, competitive adsorption arises between organic matter in the solid phase and dissolved organic carbon (DOC), generally leading to an increase in Cu concentration in the soil solution due to increased DOC concentration (Carrillo-Gonzalez et al. 2006). However, upon increasing pH, the free cupric ion activity considerably decreases at the expense of organically-bound complex species in the soil solution (Sauvé et al. 1997). This is the reason why it is often considered that the phytotoxicity of Cu is minimal in calcareous soils, assuming that plants are only sensitive to the free cupric ion.

The rhizosphere is the volume of soil around roots that is influenced by the activity of living plant roots (Hinsinger et al. 2005). In the rhizosphere, root and microbial activities can change the liquid and solid phase speciation and ultimately the mobility of metals as a consequence of alterations of chemical properties such as soil pH or DOC concentration (Hinsinger and Courchesne 2007). In the particular case of *Graminaceous* species, the increased root secretion of iron (Fe) chelating compounds under Fe deficiency, the so-called phytosiderophores, have been reported to increase Cu uptake in a calcareous soil (Chaignon et al. 2002b). In addition, root-mediated pH changes can influence the chemical mobility of nutrients and metals in the rhizosphere, and thereby their uptake by plants (Hinsinger et al. 2005). It has been shown that increasing the pH in the rhizosphere of plants grown in acidic soils can decrease aluminium (Al) concentration in the soil solution, and hence alleviate Al phytotoxicity (Marschner 1995; Kochian et al. 2004).

Copper is an essential nutrient which is known to result in phytotoxic damages such as rhizotoxicity and induced Fe deficiency when present at elevated concentrations (Marschner 1995). Numerous former studies were carried out to identify relationships between plant Cu uptake (or Cu phytotoxicity) and soil chemical properties, such as pH and various indicators of Cu contamination (Sauvé et al. 1996; Brun et al. 2001; Chaignon et al. 2003; Nolan et al. 2005; Zhao et al. 2006). Those former studies were mainly carried out under controlled, artificial conditions, such as the use of sieved, air dried and sometimes spiked soils. In contrast, to our knowledge no study has been carried out in situ to evaluate the actual plant Cu concentration in roots and shoots and environmental risks of Cu phytotoxicity as related to soil chemical properties. In addition, soil chemical properties can differ between the bulk soil and the rhizosphere, so that considering soil chemical properties in the bulk soil might be a poor predictor of Cu uptake which actually depends on the peculiar chemical properties induced by roots in the rhizosphere. The aim of this work was thus to assess in situ the uptake and phytotoxicity of Cu in durum wheat cropped in Cu-contaminated, former vineyard soils and to identify the underlying soil chemical properties while taking account of root-induced chemical changes in the rhizosphere.

Materials and methods

In situ sampling of soils and plants

Forty-two samples of Cu-contaminated, former vineyard soils and durum wheat (*Triticum turgidum durum* L.) cultivated in these soils were collected between March and May 2006 at 30 km around Béziers (Southern France, 60 km W of Montpellier). Soil samples comprised 20 calcareous and 22 non-calcareous soils and covered a large range of pH and total Cu concentration (Table 1). Plants were harvested at the stage of 1 cm spike (end of tillering stage). The whole plants including the roots were collected by sampling $20 \times 20 \times 20$ cm topsoil around a wheat row. Plants were extracted manually from the soil clods and the soil strongly adhering to the roots was operationally defined as the rhizosphere. This rhizosphere was collected by gently washing the roots

in deionised water. A subsample of fresh bulk soil was washed in deionised water during 5 min in a soil: solution ratio close to the one used to recover the rhizosphere. An aliquot of the deionised water used for washing off the soil and the rhizosphere was filtered at 0.45 μm and frozen at −20°C prior to measuring its total Cu concentration (water extractable Cu). Soil samples (bulk, washed bulk and rhizosphere) were air-dried and sieved at 2 mm prior to being used for subsequent analysis. Roots and shoots of eight plants were washed carefully with deionised water. Shoots were oven dried at 105°C and roots were frozen at −20°C.

Plant analyses

Copper bound to root cell walls (root apoplasmic Cu) was determined as described by Chaignon et al. (2002b). Briefly, after thawing, a subsample of 0.8 g of roots (on a fresh matter basis) was shaken end-over-end with 40 cm^3 of 1 mM HCl for 3 min, then 360 mm^3 of 1 M HCl were added to yield a final concentration of 10 mM HCl. After shaking for another 3 min, the suspensions were filtered through ashless filter paper (Whatman 541) and roots recovered and oven dried at 105°C. After extraction of root apoplasmic Cu, Cu remaining in roots was considered to be symplasmic Cu. The remaining portion of untreated roots was also oven dried at 105°C. The oven dried roots and shoots were digested separately in a microwave oven with hot concentrated HNO_3. Concentrations of Cu and Fe in the root and shoot digests were determined by Flame Atomic Absorption Spectrometry (Varian SpectrAA-600, Australia) and reference plant materials (BCR 62, olive (*Olea europaea* L.) leaves; BIPEA V463, maize (*Zea mays* L.) shoots) were used as standards. Root symplasmic Cu was determined by measuring Cu concentration in the root digests of roots recovered after extraction of root apoplasmic Cu.

Soil analyses

All the soil analyses that are summarised in Table 1 were carried out by a routine soil testing laboratory (INRA LAS Arras, France) according to standardised French procedures (AFNOR 1999) or international procedures (ISO 1999). Soil pH was measured in 0.01 M $CaCl_2$ using a 1:5 soil:solution ratio (ISO 10390-ISO 1999). After calcium (Ca) carbonate dissolution, the soil samples were partitioned into five particle size fractions, 0−2, 2−20, 20−50, 50−200, 200−2,000 μm. Cation exchange capacity (CEC) was determined by cobaltihexamine chloride extraction. Total Ca carbonate, total nitrogen and organic carbon concentrations were determined according to ISO (1999). Total Cu and Fe concentrations were determined by HF–$HClO_4$ extraction. The fraction of soil Cu and Fe which was potentially available to the plant was determined by using a solution of 0.01 M Na_2–EDTA (ethylene diaminotetraacetic acid)/ammonium acetate (pH 7). The Fe oxide concentrations were determined by Mehra–Jackson and Tamm (darkness) methods while the Mn oxide concentration was determined by the Deb method (Baize 2000).

The pH, water- and $CaCl_2$-extractable Cu were determined in the rhizosphere and bulk soil. Copper extracted by water was determined during the soil and root sampling process as described above. An aliquot of air-dried rhizosphere and bulk soil was shaken in a solution of 0.01 M $CaCl_2$ for 2 h using a 1:10 soil: solution ratio (Lebourg et al. 1998). The suspension was then centrifuged at 10,000 g for 10 min. After measuring the pH, the supernatant was kept at 4°C before assaying Cu concentration. Copper concentration in water extracts was determined by Graphite Furnace Atomic Absorption Spectrometry (Varian SpectrAA-600). Copper concentration in $CaCl_2$ extracts was determined by Inductively Coupled Plasma–Atomic Emission Spectrometry (Varian Vistapro).

Statistical analyses

Linear regressions (Spearman R) and analyses of variance (Kruskal–Wallis analyses) with nonparametric tests were performed to identify significant differences with Statistica® (Statsoft, version 6, Tulsa, OK, USA).

Results

Soil Cu concentration

The amounts of total Cu in soil samples varied considerably, ranging from 32 mg kg^{-1} in sample 12, which is close to normal, geochemical background level in soils, to 184 mg kg^{-1} in sample 13, for 40 out of the 42 soil samples (Table 1). The

Table 1 Selected chemical and physical properties of soils

Sample	Cu (mg kg⁻¹ soil)		pH CaCl₂	CaCO₃ (g kg⁻¹)	CEC (cmol_c + kg⁻¹)	Organic C (g kg⁻¹)	Organic N (g kg⁻¹)	Fe (g kg⁻¹)		Fe oxide (g kg⁻¹)		Mn oxide (g kg⁻¹)	Clay (g kg⁻¹) 0–2 μm	Silt 2–50 μm	Sand 50–2000 μm
	Total	EDTA						Total	EDTA	Mehra J	Tamm	Deb			
1	117	41	4.2	nd	5.8	4.5	0.58	32	0.10	18.4	2.3	0.93	223	233	544
2	88	21	4.3	nd	11.6	10.5	1.00	32	0.08	19.7	0.8	0.33	373	186	441
3	100	42	4.6	nd	3.6	3.8	0.43	16	0.05	10.9	1.2	0.75	130	174	696
4	97	44	5.3	nd	9.8	17.0	1.51	23	0.09	13.1	0.7	0.28	253	284	463
5	82	29	5.4	nd	9.8	5.7	0.61	25	0.05	14.3	1.8	0.48	260	208	532
6	72	23	5.5	nd	14.7	6.0	0.69	36	0.04	20.5	1.7	0.42	363	209	428
7	79	23	5.5	nd	14.2	11.7	1.09	31	0.04	19.4	0.8	0.49	368	199	433
8	165	64	5.6	nd	14.8	15.1	1.56	36	0.07	19.7	1.4	0.27	383	250	367
9	53	21	5.8	nd	8.8	9.1	0.92	27	0.05	17.2	1.8	0.53	280	197	523
10	94	33	5.9	nd	15.8	9.1	1.00	31	0.03	16.0	1.3	0.44	383	220	397
11	89	36	5.9	nd	8.3	5.6	0.53	20	0.04	11.4	0.7	0.39	164	239	597
12	32	14	5.9	nd	10.8	7.8	0.75	19	0.04	10.7	1.2	0.30	241	170	589
13	184	77	6.0	nd	14.6	17.6	1.69	38	0.06	20.7	2.0	0.50	270	236	494
14	123	55	6.2	nd	14.1	8.9	0.81	23	0.04	12.7	0.9	0.32	263	275	462
15	150	63	6.3	nd	10.9	15.1	1.38	29	0.05	16.1	1.3	0.58	209	219	572
16	140	81	6.3	nd	10.4	11.9	0.92	12	0.07	5.7	1.0	0.20	152	214	634
17	87	34	6.3	nd	14.3	8.7	0.92	26	0.04	13.5	1.3	0.38	323	138	539
18	141	66	6.3	nd	14.5	8.8	0.83	24	0.04	13.9	0.9	0.35	270	295	435
19	158	72	6.4	nd	16.1	18.1	1.73	31	0.05	18.6	0.8	0.27	330	284	386
20	103	60	6.9	<2	11.6	12.1	0.95	14	0.04	8.4	0.5	0.22	169	149	682
21	67	34	6.9	nd	10.9	11.1	1.05	17	0.03	9.6	0.6	0.23	200	208	592
22	152	61	7.2	nd	15.1	11.1	1.16	28	0.02	15.2	1.2	0.41	347	243	410
23	56	13	7.8	113	14.8	14.5	1.39	40	0.01	17.4	2.2	0.76	266	556	58
24	146	46	7.6	47	6.2	5.4	0.51	15	0.01	8.5	0.5	0.24	94	132	730
25	111	34	7.6	102	7.9	11.3	0.77	30	0.01	13.7	1.8	0.34	120	321	453
26	163	57	7.6	94	7.0	11.5	0.75	29	0.01	13.5	1.8	0.29	108	280	517
27	155	57	7.6	93	8.7	11.3	0.86	31	0.02	14.4	1.7	0.36	127	350	426
28	540	211	7.6	110	10.4	15.9	1.26	30	0.01	14.2	1.8	0.37	123	338	431

29	128	43	7.6	102	8.5	10.4	0.74	31	0.01	15.2	1.8	0.38	137	377	382
30	1,030	488	7.5	87	11.7	23.6	1.65	29	0.01	13.7	1.9	0.35	131	380	384
31	59	20	7.6	25	13.1	3.5	0.40	19	0.01	10.1	0.9	0.42	205	180	593
32	70	29	7.7	278	8.7	7.0	0.62	12	0.01	6.9	0.4	0.10	133	184	402
33	84	37	7.6	154	13.1	13.8	0.92	16	0.01	4.8	0.4	0.20	152	218	475
34	117	51	7.7	204	11.8	11.2	0.70	17	0.01	4.8	0.4	0.21	152	212	421
35	120	62	7.6	261	9.6	14.2	0.94	15	0.01	4.2	0.4	0.22	99	192	429
36	150	74	7.6	207	11.5	15.0	1.04	16	0.01	4.5	0.4	0.23	118	228	430
37	114	49	7.6	279	10.4	8.2	0.76	11	0.01	3.5	0.4	0.16	133	145	454
38	78	34	7.7	298	10.6	8.9	0.97	10	0.01	3.5	0.5	0.18	135	140	415
39	43	11	7.5	6	16.7	5.4	0.53	24	0.01	12.4	0.7	0.50	292	145	557
40	106	33	7.7	154	16.3	8.4	0.77	23	0.00	12.1	0.4	0.30	290	193	356
41	103	37	7.5	4	16.8	7.3	0.65	28	0.02	17.7	0.5	0.13	332	189	475
42	147	56	7.5	75	15.1	12.8	1.13	19	0.01	10.1	0.9	0.26	207	232	493

nd: not determined because non-calcareous soils.

amount of total Cu in soil samples 28 and 30 reached 540 and 1,030 mg kg^{-1}, respectively, which is fairly large compared with the other soil samples. The abnormally elevated Cu concentration in these two samples resulted from localized contamination as a result of repeated filling with Cu salts of the fungicide tank to spray vines. Copper extracted by EDTA also varied considerably from 11 to 488 mg kg^{-1} and amounted to 23–58% of total soil Cu. Soil pH varied considerably among the 42 samples, from 4.2 to 7.2 for the 22 non-calcareous soils (samples 1 to 22) and from 7.5 to 7.8 for the 20 calcareous soils (samples 23 to 42), for which the CaCO$_3$ concentration varied from 4 to 298 g kg^{-1}.

Plant Cu and Fe concentration

The Cu concentration in shoots varied from 6 to 39 mg kg^{-1} DM (Table 2 and Fig. 1a). The total and symplasmic Cu concentrations in roots varied even more so, from 11 to 705 mg kg^{-1} DM (about 66-fold) and from 6 to 150 mg kg^{-1} DM (about 25-fold), respectively (Table 2 and Fig. 1c). The symplasmic Cu amounted to 16–57% of total Cu in roots. The shoot/root ratios of Cu concentration decreased with increasing Cu concentration in soils, ranging from 0.86 for one of the least Cu-contaminated soils (sample 39) to 0.06 for the most Cu-contaminated soil (sample 30). Moreover, root Cu concentration was significantly (at $P < 0.05$) larger in calcareous soils than in non-calcareous soils for the 42 soil samples (Fig. 1c). When excluding the two most Cu-contaminated samples (samples 28 and 30) in order to compare the two types of soils in the same range of soil Cu concentration (Fig. 1d), root Cu concentration was not significantly larger in calcareous soils than in non-calcareous soils. For shoot Cu concentration, there was no significant difference between the calcareous and non-calcareous soils (Fig. 1b). Inter-veinal chlorosis symptoms (yellow stripes) along the length of the leaves (Fig. 2a) were observed in durum wheat grown in 5 of the sampled calcareous soils, either in isolated spots (samples 28 and 30) (Fig. 2b) or diffuse spots (samples 24 and 26). These were also observed along rows across the field in sample 37 (Fig. 2c). These interveinal chlorosis symptoms were particularly pronounced in the most Cu-contaminated soil which contained 1,030 mg Cu kg^{-1} (Fig. 2a). In these field plots, pairs of soil samples were collected

Table 2 Plant Cu and Fe concentration in shoots and roots, and shoot/root ratios of the Cu concentration in plant parts

| Sample	Cu concentration (mg kg^{-1} DM)			Shoot/ root ratios	Fe concentration (mg kg^{-1} DM)	
	Shoots	Roots (total)	Roots (symplasmic)	Cu	Shoots	Roots
1	11	85	29	0.31	79	1907
2	9	30	8	0.13	302	2854
3	24	74	27	0.33	149	2298
4	11	57	16	0.20	276	4201
5	7	20	11	0.32	135	2128
6	7	17	8	0.20	181	2126
7	8	26	14	0.42	266	7166
8	9	47	14	0.33	193	1943
9	7	23	12	0.31	326	5323
10	7	18	10	0.41	82	1509
11	9	36	13	0.26	134	4356
12	8	11	6	0.18	116	1078
13	11	78	16	0.32	127	1717
14	13	48	18	0.71	294	3956
15	10	55	22	0.24	364	2505
16	13	105	30	0.27	217	5444
17	9	27	13	0.14	112	2911
18	14	58	19	0.18	184	3289
19	13	53	14	0.24	169	3120
20	9	50	10	0.12	110	2961
21	13	85	21	0.15	189	6475
22	9	49	22	0.19	213	2620
23	14	29	8	0.49	271	6040
24	17	198	45	0.09	182	1531
25	11	65	14	0.17	255	4206
26	14	128	26	0.11	171	2619
27	8	56	12	0.15	277	2256
28	21	289	69	0.07	251	1217
29	7	39	10	0.19	168	2706
30	39	705	150	0.06	331	1358
31	8	26	8	0.30	119	4185
32	9	87	19	0.10	81	2715
33	8	53	9	0.15	173	1610
34	9	72	16	0.13	168	2580
35	12	80	13	0.14	156	1976
36	18	93	15	0.20	223	2103
37	11	213	57	0.05	166	2536
38	13	125	26	0.11	183	2280
39	15	17	7	0.86	193	4649
40	6	47	11	0.14	105	2404
41	10	54	14	0.19	412	3924
42	7	40	10	0.17	95	1632

where plants were exhibiting or not the interveinal chlorosis symptoms in order to test whether these were possibly linked with Cu phytotoxicity. Samples 27 and 29 were sampled in the field plot beside the spots corresponding to the 2 most Cu-contaminated soil samples, samples 28 and 30, respectively. Samples 37 and 38 were sampled in the same field plot, in one of the rows formed by durum wheat exhibiting the interveinal chlorosis symptoms and outside of these rows, respectively. The last pair of samples corresponded to samples 25 and 26 for which plants presented no symptoms and symptoms of interveinal chlorosis, respectively. In these pairs of samples, soil Cu concentration was larger in soils corresponding to plants exhibiting interveinal chlorosis symptoms (Table 1). The Cu concentrations in plants which exhibited such symptoms was larger than in plants which had no symptoms and reached 11–39 mg kg^{-1} DM in shoots (Table 2 and Fig. 2a) and 128–705 mg kg^{-1} DM in roots (Table 2 and Fig. 2c). In addition, the shoot/root Cu concentration ratios of plants exhibiting interveinal chlorosis symptoms were among the lowest, especially in samples 28, 30 and 37 (0.07, 0.06 and 0.05, respectively).

The Fe concentration in shoots varied from 79 to 412 mg kg^{-1} DM and the Fe concentration in roots varied from 1,078 to 7,166 mg kg^{-1} DM (Table 2). Moreover, the root Fe concentration tended to decrease with increasing soil Cu concentration (Fig. 3a) and root Cu concentration (Fig. 3b), particularly in calcareous soils. In contrast, the Fe concentration in shoots did not show any relationship with either soil or shoot Cu concentrations. Noteworthy, plants exhibiting interveinal chlorosis symptoms exhibited the largest root Cu concentrations, among the lowest root Fe concentrations and among the largest shoot Fe concentrations.

Relationships between plant Cu concentration and soil properties

Linear regressions were performed in order to define the relationships between Cu concentrations in plant parts (shoot Cu and root Cu), extractable soil Cu (total and EDTA–Cu) and other soil properties (pH, CEC, CaCO$_3$, organic C, total N, Fe and Mn oxides and clay concentrations). As the two most Cu-contaminated samples (samples 28 and 30) were assumed to strongly influence some of the relationships, especially between soil Cu concentration and Cu concen-
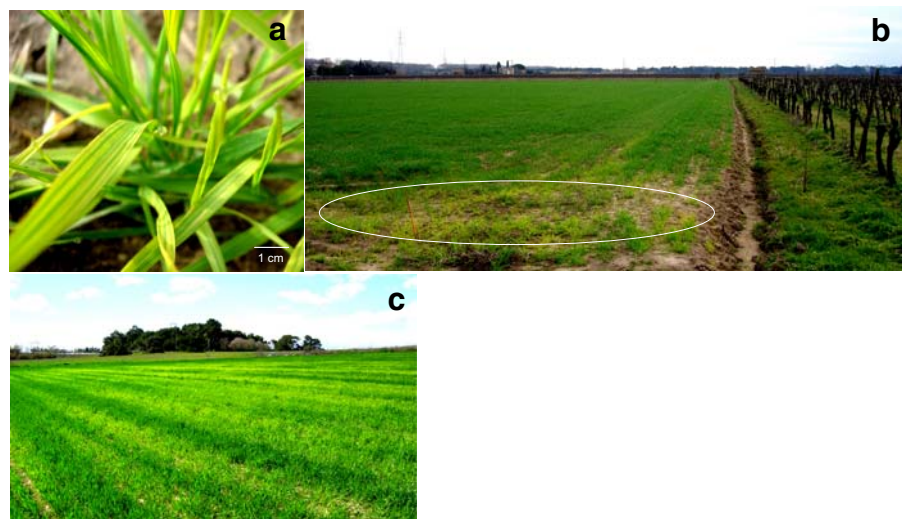
Fig. 1 Copper concentration in plant parts (mg kg^{-1} DM) as a function of Cu concentration in soil (mg kg^{-1} soil), measured in shoots (**a**, **b**) and in roots (**c**, **d**), for the 42 samples (**a**, **c**) and 40 samples (without the two most Cu-contaminated samples 28 and 30) (**b**, **d**). *Open circles* stand for plants grown in calcareous soils and *open triangles* stand for plants grown in calcareous soils and exhibiting interveinal chlorosis symptoms while *filled circles* stand for plants grown in non-calcareous soils
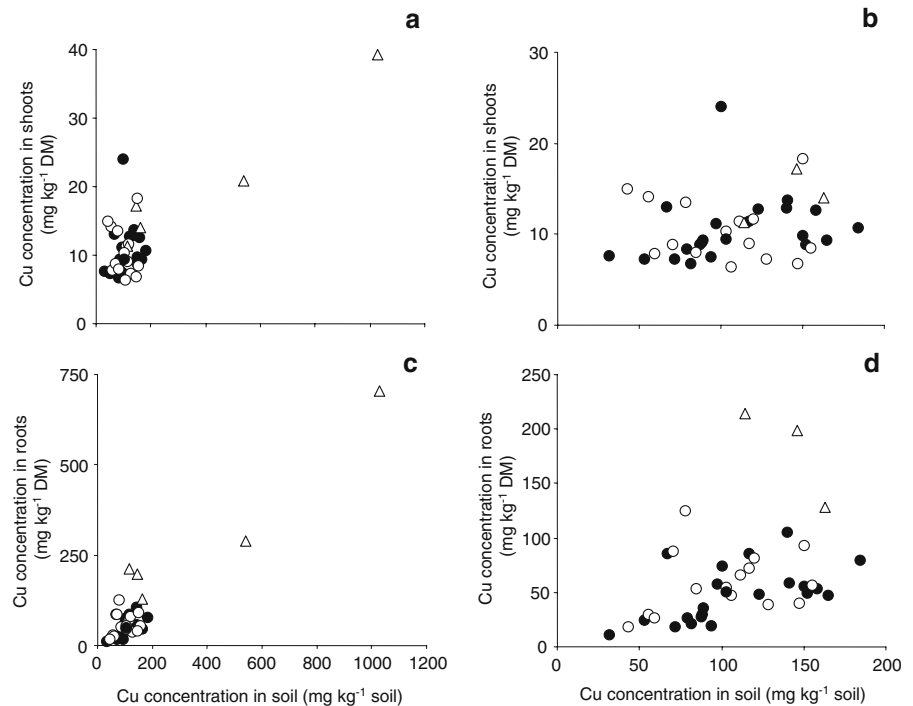
trations in plants parts, linear regressions were also performed without samples 28 and 30. Relationships between root and shoot Cu concentrations and total soil Cu concentration are shown in Fig. 1. Shoot Cu concentration was correlated to total soil Cu concentration only when considering the 42 soils (Fig. 1a)

($r=0.32$, $P<0.05$), not when excluding the two most contaminated soils (samples 28 and 30) (Fig. 1b). In contrast, a stronger relationship was found for root Cu concentration which increased with increasing total soil Cu concentration ($r=0.56$, $P<0.01$) (Fig. 1c) even when excluding the two most contaminated soils

Fig. 2 Interveinal chlorosis symptoms of alternate yellow and green stripes along the length of the leaf observed in durum wheat (**a**) cropped in the spot represented by the *white circle* where the tank for fungicide treatment of former vines was filled with Cu-salts (sample 30) (**b**) and such symptoms observed in durum wheat along rows across the field (sample 37) (**c**)
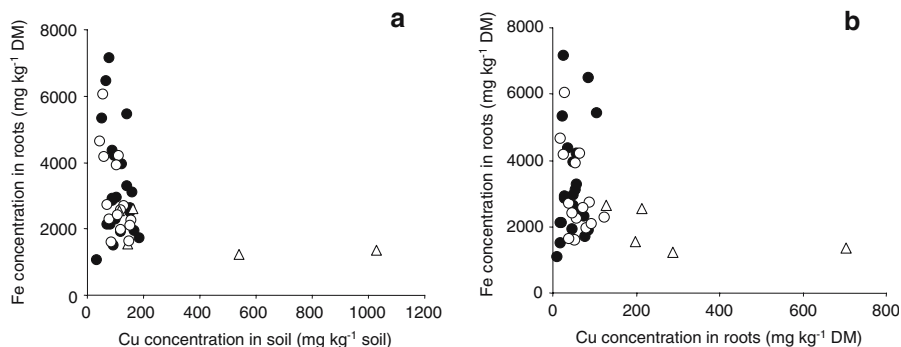
Fig. 3 Iron concentration in roots (mg kg^{-1} DM) as a function of Cu concentration in soil (mg kg^{-1} soil) (**a**) or Cu concentration in roots (mg kg^{-1} DM) (**b**) for the 42 samples. *Open circles* stand for plants grown in calcareous soils and

open triangles stand for plants grown in calcareous soils and exhibiting interveinal chlorosis symptoms while *filled circles* stand for plants grown in non-calcareous soils

($r=0.49$, $P<0.01$) (Fig. 1d). In addition, Cu concentration in shoots and roots were positively correlated to EDTA extractable Cu ($r=0.42$ and 0.65, respectively, $P<0.01$) for the 42 samples, even when excluding the 2 most Cu-contaminated soils ($r=0.34$, $P<0.05$ and 0.59, $P<0.01$, respectively), Soil pH was positively correlated to root Cu concentration ($r=0.33$, $P<0.05$), even when excluding the two most Cu-contaminated soils ($r=0.32$, $P<0.05$). Conversely, organic carbon concentration was not correlated to both shoot and root Cu concentrations. Nevertheless, it was positively correlated to total soil Cu concentration ($r=0.54$, $P<0.01$) for the 42 soils and when excluding the 2 most Cu-contaminated soils ($r=0.47$, $P<0.01$). Moreover, root Cu concentration appeared negatively correlated to clay content ($P<0.01$), CEC ($P<0.01$), Mehra–Fe oxides ($P<0.05$) and Mn oxides ($P<0.01$), while it appeared positively correlated to CaCO$_3$ concentration ($P<0.01$), even when excluding the two most Cu-contaminated soils.

Chemical changes in the rhizosphere

Soil pH changes in the rhizosphere were negatively correlated to bulk soil pH ($r=-0.83$, $P<0.01$) (Fig. 4). In non-calcareous soils, a significant ($P<0.05$) increase in rhizosphere pH was observed with decreasing soil pH. Rhizosphere alkalization was particularly pronounced in the most acidic soils (bulk soil pH equal or below 4.6 in samples 1–3) as it reached 0.8–1.1 pH units. In contrast, no significant change of rhizosphere pH was observed in the calcareous soils, as expected due to their large pH buffering capacity.

The amount of Cu extracted by water varied from non-detectable values (detection limit of 3 µg dm^{-3}) to 1.2 mg kg^{-1} in the bulk soil and from non-detectable values to 11.1 mg kg^{-1} in the rhizosphere (Fig. 5a). Amounts of Cu extracted by water were thus significantly ($P<0.01$) larger in the rhizosphere whatever the bulk soil pH. The largest amounts of Cu extracted by water were observed in the rhizosphere of either those plants growing in the most acidic soils (samples 1 and 3) or to those plants exhibiting interveinal chlorosis symptoms (samples 28, 30 and 37, triangles in Fig. 5a) combined with the largest root Cu concentrations. The amounts of Cu extracted by water in
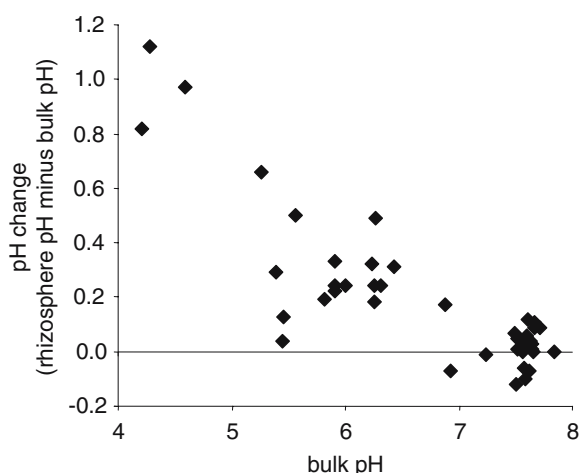

Fig. 4 Soil pH change (rhizosphere pH minus bulk soil pH) as a function of bulk soil pH (pH values measured in the CaCl$_2$ extract)
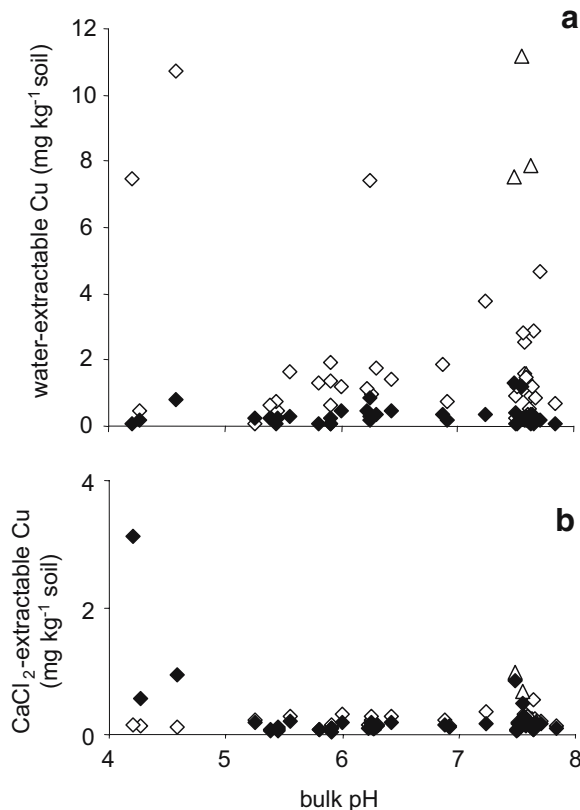
Fig. 5 Copper extracted by water (mg kg^{-1} soil) (**a**) and Cu extracted by CaCl$_2$ (mg kg^{-1} soil) (**b**) as a function of the pH measured in the bulk soil. *Open diamonds* stand for Cu extracted in the rhizosphere and *open triangles* stand for Cu extracted in the rhizosphere of plants exhibiting interveinal chlorosis symptoms while *filled diamonds* stand for Cu extracted in the bulk soil

the rhizosphere presented the largest values and were significantly ($P<0.01$) larger than the amounts of Cu extracted by CaCl$_2$. Indeed, the amounts of Cu extracted by CaCl$_2$ varied from 0.06 to 3.1 mg kg^{-1} in the bulk soil and from 0.07 to 1.0 mg kg^{-1} in the rhizosphere (Fig. 5b). The amounts of Cu extracted by CaCl$_2$ were slightly larger in the rhizosphere than in the bulk soil for 38 samples, but no significant difference was observed. In contrast, for four samples including the three most acidic soils (samples 1–3) the amounts of Cu extracted by CaCl$_2$ were by far lower in the rhizosphere, compared with the bulk soil. Indeed, at bulk soil pH below 4.6, Cu extracted by CaCl$_2$ reached 0.6–3.1 mg kg^{-1} in the bulk soil, while it amounted to only 0.1–0.2 mg kg^{-1} in the rhizosphere.

Discussion

Plant Cu uptake and Cu phytotoxicity in field-grown plants

In spite of the large range of total Cu concentration in the 42 investigated soils, Cu concentration in shoots varied within a narrow range, while root Cu concentration varied considerably (Table 2 and Fig. 1). The proportion of Cu actually absorbed by roots, i.e. symplasmic Cu, represented between 16–57% of total root Cu concentration. This confirmed for field-grown plants the results obtained in former studies conducted in the laboratory suggesting that the major part of root Cu was adsorbed onto root cell walls (Chaignon et al. 2002b), notably in calcareous soils as their high pH is prone to enhanced adsorption. With increasing soil Cu concentration, plants appeared capable of restricting Cu translocation towards their aerial parts, particularly in plants exhibiting interveinal chlorosis symptoms (shoot/root ratios in Table 2). As stressed in former studies, root Cu concentration was by far more sensitive to soil Cu contamination and thus appeared as a better indicator of Cu contamination than shoot Cu concentration presumably due to efficient control of Cu translocation from roots to shoots (Brun et al. 2001; McBride 2001; Chaignon and Hinsinger 2003; Chaignon et al. 2003).

Copper concentrations in plants exhibiting interveinal chlorosis were larger, particularly in roots, than in plants which did not display such symptoms (Table 2 and Fig. 2). Such interveinal chlorosis symptoms in leaves of cereals are widely recognized as indicative of lime-induced Fe deficiency (Marschner 1995; Mengel and Kirkby 2001). When comparing pairs of samples of durum wheat exhibiting or not such interveinal chlorosis as collected in nearby locations in the same field plots, larger soil Cu concentration in samples 26, 28, 30 and 37 occurred to be related to larger plant Cu concentrations and Fe deficiency symptoms, compared with samples 25, 27, 29 and 38. Several studies with hydroponically-grown plants have reported that Cu phytotoxicity can result in chlorosis as a consequence of induced Fe deficiency (Lexmond and Van der Vorm 1981; Wheeler and Power 1995; Kopittke and Menzies 2006). In addition, McBride (2001) observed interveinal chlorosis symptoms in maize grown in Cu-spiked soils. Historical Cu contamination of vineyard soils seems to have resulted in Cu accumulation in

plants, and hence in Fe deficiency chlorosis symptoms presumably related to Cu phytotoxicity.

Interaction between Fe nutrition and Cu phytotoxicity

Root Fe concentration decreased with increasing soil Cu concentration (Fig. 3a) and with increasing root Cu concentration (Fig. 3b), especially for calcareous soils for which even when excluding the two most contaminated soils (samples 28 and 30) such decrease was still noticeable. Moreover, interveinal chlorosis symptoms coincided with low root Fe concentration, especially in the two most Cu-contaminated soils (Table 2). This suggested an antagonistic relation between Cu and Fe which may have led to impaired Fe uptake and to decreased Fe concentration in roots. In contrast, no such relationship was observed for shoot Fe concentration (Table 2). It has been reported that leaves exhibiting Fe deficiency symptoms may have larger total Fe concentrations than control leaves, probably because of Fe immobilization in the leaf apoplast (Sattelmacher 2001). Moreover, former studies showed that metabolically active Fe in leaves was better predictor of the Fe status compared with total Fe (Lang and Reed 1987). Determining active Fe in shoots may thus have led to ascertain whether interveinal chlorosis was due to low Fe activity as a result of Cu phytotoxicity. In addition, it has been shown in bread wheat that Cu uptake increased under Fe deficiency in a Cu-contaminated, calcareous soil as related to increased secretion of phytosiderophores (Chaignon et al. 2002b). In such soils, increased secretion of phytosiderophores might have increased the Cu uptake by plants at the expense of Fe uptake (Reichman and Parker 2005), so that symptoms of Cu phytotoxicity and induced Fe deficiency coincided, as shown by the interveinal chlorosis observed in situ.

Plant Cu concentration versus soil properties

Among soil chemical properties, soil pH, organic carbon concentration and soil Cu concentration are expected to influence the solid phase speciation of Cu (McBride 1981) and hence, Cu bioavailability. The EDTA extractable fraction of a metal is considered as the environmentally available fraction, i.e. the fraction which is potentially available for soil organisms (Harmsen et al. 2005). In the present work EDTA extractable Cu was indeed slightly better correlated to

plant Cu concentration than total soil Cu. Our results, in spite of having been obtained in situ, are in line with those of Brun et al. (2001), Chaignon et al. (2003) and Nolan et al. (2005) who reported in pot experiments that total Cu concentration could surprisingly be an adequate predictor of plant Cu concentration, especially for root Cu concentration (Fig. 1). Nevertheless, poor linear correlation coefficients between plant Cu concentration and soil Cu concentration were obtained especially for mild soil Cu-contamination. This may be explained by sources of variations among the studied sites, and hence of factors influencing plant growth and Cu uptake which are manifold and include for instance variations in microclimate, agricultural practices and durum wheat cultivars. In addition, more evenly distributed soil Cu concentrations in the investigated range would have possibly improved the relationships between soil Cu concentration and plant Cu concentration, or at least help to ascertain these.

Literature reported contradictory results concerning the effect of pH on Cu uptake by plants. In very acidic soils, plant Cu concentration increased compared to calcareous soils in rape (Brassica napus L.) and tomato (Lycopersicon esculentum L.) (Chaignon et al. 2002a, 2003; Cornu et al. 2007). Conversely, Cu accumulation in maize was as high in calcareous soils as in acid soils (Brun et al. 2001) and, according to Zhao et al. (2006) increasing soil pH may even increase Cu toxicity at a given free Cu^{2+} activity. The apparent paradox of increasing Cu toxicity with increasing pH, presumably due to decreased competition between proton and Cu for adsorption onto root cell walls, is partly explained by the Terrestrial Biotic Ligand Model (TBLM) which accounts for the effects of competing cations (proton, magnesium, calcium) at the biotic ligand sites and has been shown to adequately predict Cu toxicity (Thakali et al. 2006a, b). In the present study, no clear relationship between plant Cu uptake and soil pH was found. Nevertheless, root Cu concentration was positively correlated to soil pH and was significantly larger in calcareous soils than in non-calcareous soils when considering the two most Cu-contaminated soils (samples 28 and 30), suggesting that root Cu concentration tended to increase with increasing pH. In addition, Fe chlorosis symptoms probably related to Cu phytotoxicity were observed only in calcareous soils. This suggests that plant Cu uptake and risks of Cu phytotoxicity might

be greater in calcareous soils than in non-calcareous soils. However, the investigated range of soil Cu concentration in non-calcareous soils may have been too low to induce visible symptoms of Cu phytotoxicity. In addition to the abovementioned symptoms associated with induced Fe deficiency, it is well known that the primary symptom of Cu toxicity in plants is rhizotoxicity, which results in reduced root elongation (Kopittke and Menzies 2006). But, the sampling method employed in the present work was not suited to recover the whole root system and reliably measure root length.

Root-induced changes in the rhizosphere of field-grown plants

Soil properties can differ between the bulk soil and the rhizosphere, so that chemical properties of the bulk soil might be poor predictor of Cu bioavailability which rather depends on the peculiar properties of the rhizosphere (Hinsinger and Courchesne 2007). Alkalization occurred at low pH in the rhizosphere compared with the bulk soil (Fig. 4). In non-calcareous soils, such rhizosphere alkalization had been observed in pot experiment by Chaignon et al. (2002a), particularly at pH below 5.5. In the present work which reports on samples collected in field-grown plants, the large alkalization (0.8–1.1 pH unit) observed in the rhizosphere for the most acidic soils (samples 1–3) might have resulted from tolerance mechanism to low pH and resulting aluminium (Al) toxicity (Kochian et al. 2004). Such alkalization can affect rhizosphere chemistry, alleviate Al toxicity and similarly reduce Cu availability.

Copper extractability was indeed altered in the rhizosphere (Fig. 5). Water extractable Cu is supposed to represent dissolved Cu in the soil solution (Courchesne et al. 2006) and $CaCl_2$ extractable Cu represents exchangeable Cu (Chaignon et al. 2002a). Copper extracted by water was significantly larger in the rhizosphere than in the bulk soil whatever the soil pH (Fig. 5a). Conversely, amounts of Cu extracted by $CaCl_2$ in the most acidic soils (samples 1–3) were by far larger in the bulk soil (about 4- to 18-fold larger). It has been shown that alkalization in the rhizosphere of tomato resulted in a reduction in the solubility of soil Cu (Cornu et al. 2007). In the present work, rhizosphere alkalization in the most acidic soils might have reduced $CaCl_2$ extractability but not water extractability of Cu.

The percentage of free Cu^{2+} has been shown to decrease with increasing pH and due to larger DOC (Vulkan et al. 2000). Actually, the major fraction of total soil solution Cu is usually present as a range of Cu-organic complexes, particularly at pH levels above 5 (Römkens et al. 1999). Former studies have reported larger DOC concentration in the rhizosphere compared with the bulk soil and have concomitantly observed larger amounts of dissolved Cu in the rhizosphere than in the bulk soil (Cattani et al. 2006; Courchesne et al. 2006). In addition, it has been observed that Ca-induced coagulation of organic matter might lead to underestimate Cu bound to organic matter in $CaCl_2$ extracts (Sauvé et al. 1997). In the present study, greater water extractability of Cu in the rhizosphere, compared with $CaCl_2$ extractability, could thus result from greater DOC concentration in the rhizosphere and presumably from larger concentration of dissolved organic substances able to mobilize Cu from the solid phase by complexation. Indeed, cereals release phytosiderophores (Reichman and Parker 2007), which have been shown to contribute to Zn and Cu mobilization in calcareous soils especially as a response to Fe deficiency (Treeby et al. 1989; Chaignon et al. 2002b). In calcareous soils which are prone to induce Fe deficiency, the release of phytosiderophores might thus explain the greater water extractability of Cu observed in the rhizosphere, compared with the bulk soil, which was particularly elevated in the rhizosphere of those plants which exhibited Fe deficiency symptoms (interveinal chlorosis) and the largest root Cu concentration (samples 28, 30 and 37). This may explain the increased uptake of Cu by plants found in these soils leading to Fe chlorosis. Degryse et al. (2006) observed that at a given free Cu^{2+} activity, Cu uptake was less in weakly buffered solutions than in solutions strongly buffered by soluble Cu complexes. In calcareous soils, larger amount of soluble organically bound Cu may serve as Cu buffer and lead to greater Cu uptake and phytotoxicity. Actually, the calcareous soils in which root Cu concentration and Cu phytotoxicity occurred had relatively high organic C contents (samples 26, 28 and 30). The buffering effect of DOC in the soil solution of calcareous soils may therefore be another reason for greater Cu phytotoxicity. Moreover, increased root exudation of organic anions as Cu- and Al-resistance mechanism was shown in various species, including wheat (Rengel 1996; Ma et al.

2001). In very acid soils, such increase in organic anion exudation could have resulted in Cu mobilization from the solid phase, leading to larger water extractability of Cu and to lower concentration of free Cu^{2+} species in soil solution due to complexation with organic anions, ultimately resulting in lower Cu uptake by plants despite the strong acidic pH of the bulk soil.

In conclusion, our results showed that total soil Cu and EDTA extractable Cu could be reasonable predictors of Cu concentrations in field-grown plants, especially in elevated Cu-contaminated, former vineyard soils. This work revealed that plant Cu uptake and even risks of Cu phytotoxicity (interveinal chlorosis) as observed in situ might be larger in calcareous soils than in non-calcareous soils due to a possible antagonism between Cu and Fe and larger DOC in soil solution. In addition, our results suggest the implication of root-induced changes of pH and DOC in the rhizosphere, leading to changes in solid phase speciation of Cu in the rhizosphere relative to the bulk soil, with potentially larger chemical mobility in the rhizosphere, as shown by the larger water extractability of Cu in the rhizosphere. In the most acidic soils, the large root-induced alkalisation may result in reduced Cu bioavailability. In the calcareous soils, the larger chemical mobility may be related to phytosiderophore secretion leading to greater Cu uptake in plants exhibiting Cu phytotoxic symptoms (interveinal chlorosis). Nevertheless, further experiments in controlled conditions are needed to demonstrate the implication of these root-induced chemical changes (e.g. pH, DOC) in the rhizosphere of cereals in Cu acquisition and phytotoxicity.

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