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Effect of Inorganic and Organic Copper Fertilizers on Copper Nutrition in *Spinacia oleracea* and on Labile Copper in Soil

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ABSTRACT: To ensure an optimal concentration of Cu in food crops, the effectiveness of eight liquid Cu fertilizers was studied in a spinach (*Spinacia oleracea* L.) crop grown on Cu-deficient soil under greenhouse conditions. Plant dry matter yields, Cu concentrations in spinach plants (total and morpholino acid (MES)- and ethylenediaminedisuccinic acid (EDDS)-extractable), and Cu uptakes were studied. The behavior of Cu in soil was evaluated by both single and sequential extraction procedures. The highest quantities of Cu in labile forms in the soil, total uptakes, and Cu concentrations in the plants were associated with the application of the two sources that contained Cu chelated by EDTA and/or DTPA. The fertilizers containing these Cu chelates represent a promising approach to achieve high levels of agronomic biofortification. The stronger correlations obtained between low-molecular-weight organic acid-extractable Cu in soil and the Cu concentrations and Cu uptakes by the plants show the suitability of this soil extraction method for predicting Cu available to spinach plants.

KEYWORDS: *availability, copper deficiency, agronomic biofortification, fertilizers, spinach*

INTRODUCTION

Copper is an essential metal for plants and plays key roles in photosynthetic and respiratory electron transport chains.[1] Soil Cu deficiencies occur in many areas of the world and can cause drastic reductions in crop yields.[2,3] Furthermore, Cu deficiencies in humans resulting from foodstuffs derived from plants and animals exposed to low Cu levels are more of a concern. The diets of much of the world's population provide Cu at just above the lower limit of the currently recommended dietary allowance (RDA).[4,5] Vegetables, such as spinach, provide a significant proportion of Cu, especially for people whose diets include only small amounts of fish or meat. Increasing the Cu concentration and Cu bioavailability − the degree to which an absorbed nutrient is available to the living system − in food crops by applying fertilizers at levels in excess of those required for maximum yield can result in significant increases in their concentrations in edible plant products (agronomic biofortification), which may help to prevent micronutrient deficiencies in humans.[4,6−8] High responses to Cu have been observed in a wide range of agricultural crops[8] such as cereals, fruits and some vegetable crops, e.g. spinach which has one of the highest per capita consumption rates in developed countries among leafy vegetable crops.[9]

Soils vary widely in their micronutrient contents and in their ability to supply sufficient micronutrients for optimal crop growth.[10] There is a general consensus in the literature that measuring the total concentration of metals in soils usually provides inadequate information to permit an assessment of their availability to plants.[11,12] Soil tests and plant analyses are tools for monitoring the micronutrient status of soils and crops. One of the most widely used approaches in soils for assessing potentially available trace elements to plants is the one-step chemical extractions. The most widely used extractant for micronutrient cations is 0.05 M diethylenetriaminepentaacetic acid (DTPA) in combination with triethanolamine (TEA),[13] but other reagents such as low-molecular-weight organic acids (LMWOAs) are recently used to estimate availability. These acids are important plant root exudates and microbial metabolites present in soils which can influence metal solubility in soils and therefore metal uptake by plants due to their chelating and/or complexing properties. Feng et al.[14,15] recommended the use of rhizosphere soil in combination with a mixture of 0.01 M LMWOAs as an extractant for evaluating in soils the availability of metals to plants. To date, the ability of this extraction method to estimate metal uptake has only been demonstrated in relatively few studies.[12,16,17] Despite substantial efforts, no single method has been universally recognized and the ones used tend to be only useful under certain specific conditions.[2,18] The amount of easily leachable metal in soils can also be estimated by means of a single chemical extraction using $BaCl_2$ as the extractant.[19] According to Räisänen et al.,[20] the $BaCl_2$ reagent extracts only elements that are physically adsorbed onto particles. The use of sequences of different chemical reagents has become an increasingly popular method for quantifying the amounts of metals present in soils, in their different fractions. The sequential extraction procedures (SEP) provide knowledge about the affinity of Cu to soil components and the strength with which it is bound to the matrix. The SEP also give information about both mobile and stabile fractions of Cu in soil, which is important for evaluating both the actual and potential transport of Cu.[21] The common definition of labile metals is defined in terms of metals that exist in solid phases in equilibrium with the pore water.[22] According to this definition,

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LMWOA- and $BaCl_2$-extractable Cu and WS-Cu could be considered labile forms in soil.

The plant parameter most frequently used to determine the Cu nutritional status of the crops is the total concentration of the micronutrient in plant dry matter (DM), although a better indication is sometimes obtained by simply determining a fraction of the total content, such as the part that is soluble in water or in diluted acids or chelators.[23] For most crops, above ground vegetative tissues have been sampled; however, some studies suggest that whenever possible, leaves should be sampled to characterize the micronutrient status of the crops.[8] Sampling young leaves for Cu may offer an added advantage because the differences in Cu concentrations between deficient and sufficient plants are greater in leaves than in stems or whole shoots and also because there is no genotype−Cu interaction in young leaves.[24] According to White and Broadley,[4] the variation in the concentration of Cu in the leaf tissues of the spinach crop was 2.2−11.0 mg kg^{-1} DM in a growth chamber trial. For other authors, the normal range of Cu concentrations in the youngest mature leaves (4−6 weeks old) were reported to range from 5 to 20 mg kg^{-1} DM for spinach.[25,26]

Although various authors have studied the response of crops to Cu fertilization, most of these studies have been conducted to examine the yield response of cereals to the incorporation of Cu fertilizers, and particularly copper sulfate and copper oxysulfate.[3,27−29] Limited information is available regarding the addition of Cu to deficient soils from other sources and for other crops. A great variety of fertilizers is available to correct soil Cu deficiencies. The level of availability of Cu and other nutrients, such as N and Fe, should be taken into consideration for equilibrated plant growth.[30,31] Commercial Cu fertilizers are available in the form of inorganic compounds, synthetic chelates and other organic complexes.[32,33] These vary considerably in their physical state, chemical reactivity, availability to plants and cost. For other micronutrients, such as Fe and Zn, various authors have shown that synthetic chelates are most efficient in calcareous soils and under hydroponic culture conditions. However, chelates are usually more expensive than other alternatives.[34−36]

The hypothesis is that Cu fertilization may or may not increase both crop yield and micronutrient concentrations in plants, depending on the soil type, crop, and fertilizer. It is therefore necessary to develop specific agricultural measures for a particular soil−crop−fertilizer combination that will be effective in increasing crop yields and the plant concentrations of a particular micronutrient. In relation to all of this, a greenhouse experiment was conducted on a Cu-deficient soil to achieve the following objectives: (i) optimize the application to soil of eight commercial Cu fertilizers (inorganic and organic) on a spinach crop aimed at enhancing DM yield and total and soluble Cu concentrations in plants; (ii) assess effects of Cu applications on the potential availability, easily leachable, and Cu distribution in fractions of soil; and (iii) elucidate the relationships between soil Cu extractability by different chemical extraction methods and uptake in a soil−spinach system.

■ MATERIALS AND METHODS

Soil Characterization. Surface soil was taken from the A_p horizon (0−20 cm) of a soil from Serracines, Madrid, Spain (latitude 40° 37′ N, longitude 3° 23′ W). This was an agricultural soil characterized by having a significant sand content and little organic matter. It was classified as a Typic Xerorthents.[37] The soil was air-dried, and a fraction of <2 mm was used in the experiment. Its main properties included the following: texture (USDA), sandy loam; clay, silt, and sand contents, 200, 150, and 650 g kg^{-1}, respectively; water-holding capacity (33 kPa), 19.7% w/w; bulk density, 1.21 g cm^{-3}; pH_w (1:1 w/v), 7.09; electrical conductivity, 35.2 μS cm^{-1} (1:2 w/v); oxidizable organic matter, 12.9 g kg^{-1}; total N, 0.45 g kg^{-1}; available P, 23.37 mg kg^{-1}; cation-exchange capacity, 9.30 $mmol_+$ kg^{-1}; base saturation, 12%. The total Cu concentration in the original soil was 9.46 mg kg^{-1}. According to the textural class of the soil, the DTPA-TEA-extractable Cu concentration is deficient for many crops (<0.4 mg kg^{-1}).[38] The analytical procedures used are described in the *Methods of Soil Analysis* manual.[39] The values presented are means of three replicates.

Applied Fertilizers. Eight liquid fertilizers with different Cu sources were selected: one was inorganic [copper oxychloride (Cu-OXYCL) (298.0 g water-soluble Cu L^{-1} and mass density = 1.65 g cm^{-3})], three were synthetic chelates [copper *N*-2-hydroxyethylethylenediaminetriacetate (Cu-HEDTA) (104.8 g water-soluble Cu L^{-1} and mass density = 1.30 g cm^{-3}); copper ethylenediaminetetraacetate (Cu-EDTA) (124.2 g water-soluble Cu L^{-1} and mass density = 1.35 g cm^{-3}); copper diethylenetriaminepentaacetate-*N*-2-hydroxyethylethylenediamine-triacetate-ethylenediaminetetraacetate (Cu-DTPA-HEDTA-EDTA) (Cu-D-H-E) (100.0 g water-soluble Cu L^{-1} and mass density = 1.31 g cm^{-3})] and four were other organic complexes [copper lignosulfonate (Cu-LS) (120.0 g water-soluble Cu L^{-1} and mass density = 1.34 g cm^{-3}); copper gluconate (Cu-GLU) (65.0 g water-soluble Cu L^{-1} and mass density = 1.30 g cm^{-3}); copper galacturonate-monogluconate (Cu-GA-MGLU) (77.0 g water-soluble Cu L^{-1} and mass density = 1.33 g cm^{-3}); copper bis(ethoxydihydroxydiethylamino)sulfate (Cu-ORG) (75.4 g water-soluble Cu L^{-1} and mass density = 1.30 g cm^{-3})].[33]

Greenhouse Pot Experiment. Nine kilograms of air-dried soil was placed in polyethylene pots (or containers, each with a capacity of 10 L, an internal diameter of 20 cm, and a height of 25 cm). The containers were placed in a greenhouse in which temperatures ranged from 12 to 28 °C and relative air humidity ranged from 60 to 85%. The pots were irrigated with appropriate amounts of water to reach and approximately maintain conditions at 60% water-holding capacity. The containers were weighed (balance A&D Instruments Ltd., UK; model FG-30 KBM) to evaluate evapotranspiration and we estimated the volume of irrigation water required. Basal fertilization was applied with 15 mg N kg^{-1} (as NH_4NO_3), 30 mg P kg^{-1} (as KH_2PO_4), and 38 mg K kg^{-1} (as KH_2PO_4 and K_2SO_4). An additional dose of 15 mg N kg^{-1} (as NH_4NO_3) was added 30 days after seed sowing. The eight liquid fertilizers were applied to the soil surface with the irrigation water to obtain samples of each with added Cu concentrations of 0 (control) and 1, 2, and 3 mg Cu kg^{-1} soil (approximately 3, 6, and 9 kg Cu ha^{-1}, respectively). These application rates are greater than typically recommended applications bearing in mind that applying fertilizers at levels in excess of those required for maximum yield can result in increasing the Cu concentration in spinach plants. The control and the different fertilizer treatments were replicated three times according to a randomized complete block design and a factorial arrangement treatment structure. Eight spinach seeds (*Spinacia oleracea* L. var. Viroflay Esmeralda; Fito S.A., Barcelona, Spain) were sown in each container and thinned to four seedlings per container after 2 weeks. Sixty days after seeding, samples of young leaves were collected. Soil samples (3 kg of air-dried soil) were taken from the upper layer of the soil in the pots (approximately 0−8 cm), as this is the area of crop root development. They were then air-dried, homogenized, sieved (<2 mm), and stored for further analysis. The plants were washed in deionized water, and then whole aerial parts were dried in a forced-draft oven at 65 °C to a constant weight. Once weighed, they were ground and kept in sealed containers for later analysis.

Plant and Soil Chemical Analyses. The total Cu concentrations in the whole aerial part of the plant DM were determined by wet acid digestion (HNO_3 + HCl) in Teflon bombs in a microwave oven (CEM Corp., model Mars, Matthews, NC, USA). The soluble Cu concentrations in fresh matter (FM) were determined from young

Table 1. Response of a Spinach Crop to Rates and Forms of Cu Sources Applied to Soil[a]

			soluble Cu in FM of young leaves		
treatment	Cu rate (mg kg^{-1})	DM yield (g pot^{-1})	MES-extractable (mg kg^{-1})	EDDS-extractable (mg kg^{-1})	total Cu in DM of whole shoots (mg kg^{-1})
control	0	2.45	0.86	1.01	9.55
Cu-OXYCL	1	4.09	1.07	1.19	12.17
	2	4.25	1.33	1.68	13.08
	3	4.47	1.76	2.46	15.28
Cu-LS	1	3.03	1.02	1.19	18.13
	2	3.47	1.28	1.41	24.44
	3	3.89	1.46	1.62	28.72
Cu-GLU	1	3.28	1.02	1.26	14.12
	2	3.69	1.13	1.37	15.59
	3	4.31	1.42	1.65	17.44
Cu-GA-MGLU	1	3.97	1.08	1.83	15.22
	2	4.29	1.46	2.06	16.77
	3	4.49	1.98	2.49	19.28
Cu-ORG	1	2.87	1.19	1.69	15.70
	2	3.60	1.76	2.21	21.92
	3	3.76	1.91	2.60	25.94
Cu-HEDTA	1	3.82	1.29	1.74	15.81
	2	4.21	1.92	2.18	18.52
	3	4.97	2.08	2.62	23.64
Cu-EDTA	1	4.21	1.65	1.88	24.21
	2	4.09	2.07	2.65	35.49
	3	3.77	4.19	4.42	52.51
Cu-D-H-E	1	4.33	1.34	2.12	23.83
	2	4.30	2.18	2.90	31.21
	3	4.26	3.55	4.12	48.17
LSD[b] (0.05)		0.32	0.42	0.42	2.63
orthogonal contrasts:[c]					
EDTA and D-H-E vs HEDTA		−0.95	4.40***	5.01***	99.49***
EDTA and D-H-E vs other Cu sources		4.71**	18.79***	21.0***	314.50***
HEDTA vs OXYCL, LS, GLU, GA-MGLU, and ORG		7.55***	5.60*	6.01**	16.04
OXYCL vs LS, GLU, GA-MGLU and ORG		6.60***	−0.02	−0.07	−71.14***

[a] Significant differences between treatments for all parameters were at $P < 0.0001$. FM, fresh matter; DM, dry matter; MES, morpholino acid; EDDS, ethylenediaminedisuccinic acid. [b] Least significant differences between treatments at $P \leq 0.05$. [c] Contrast values are the difference between treatment means: ***, **, and * denote significance at 0.00001, 0.0001, and 0.001, respectively.

leaves, using two different diluted acids: 10^{-3} M morpholino acid [MES, 2-(*N*-morpholino)ethanesulfonic acid] to pH 6[35,40] and 10^{-3} M ethylenediaminedisuccinic acid (EDDS) to pH 6. The soil samples were also digested in a microwave oven equipped with a rotating tray to measure total Cu content using an acid mixture (HNO_3 + HF). This involved a two-step process at a maximum pressure of 170 psi. Two one-step extraction procedures were used to assess the plant available Cu. First, the DTPA-TEA extraction method was applied:[13] 10.0 g of soil in 20 mL of a combined solution (5 mM DTPA + 0.01 M $CaCl_2$ + 0.1 M TEA, adjusted to pH 7.3). Second, a slightly modified version (the soil to extractant ratio was reduced to 1:4 to increase analytical precision) of the rhizosphere-based extraction method was applied (LMWOA extraction method):[15] 5.00 g of soil in 20 mL of a mixture of LMWOAs (10 mM combined organic acid solution of acetic, lactic, citric, malic, and formic acids in a molar ratio of 4:2:1:1:1, respectively). The easily leachable Cu fraction in the soil was extracted using $BaCl_2$ as reagent (3.00 g of soil in 30 mL of 0.01 M $BaCl_2$).[19] Copper distribution in the different soil fractions was determined by the SEP proposed by Pietrzak and McPhail.[41] The fractions were sequentially determined in seven steps (using a 2.5 g soil sample and a soil:extractant ratio 1:10): water-soluble (WS)−double-deionized water; exchangeable (EX)−1 M $MgCl_2$; sorbed (SORB)−1% NaCaHEDTA in 1 M NH_4Oac; easily reducible (ERMn)−0.2% $C_6H_4(OH)_2$ (hydroquinone) in 1 M NH_4OAc; organically bound (OM)−H_2O_2; bound to Fe and Al oxides −amorphous and crystallines− (FeOx)−0.175 M $(NH_4)_2C_2O_4$−0.1 M $H_2C_2O_4$; and residual fraction (RES), which was calculated as the difference between total Cu and the sum of the preceding fractions. After each succesive extraction, the soil suspension was centrifuged (4500 g for 15 min) and the supernatant obtained was filtered through 0.45 μm cellulose acetate paper and acidified with HNO_3. The Cu concentrations in all of the extracts obtained were determined using flame/graphite furnace atomic absorption spectrometry (Perkin-Elmer, AAnalyst 700). "Perkin-Elmer Pure" standard checks were used for the Quality Assurance System (certified by NIST-SRM). Standard solutions of Cu were prepared for each extraction in a background solution of the extracting agents.

Statistical Analysis. Descriptive regression analyses and other statistical studies were made using Statgraphics-Plus 5.1 software (Manugistic, Inc., Rockville, MD, USA). Regression analyses were carried out in all cases using the mean values obtained for each of the treatments studied ($n = 25$). Multifactor analyses of variance of the different parameters were carried out to determine the main effects of the Cu source, Cu rate, and experimental repetition and the interactions between them. Due to the existence of a highly significant interaction between the Cu source and Cu rate factors, we performed a new multifactor analysis of variance to determine the main effects of the fertilizer treatment (Cu source × Cu rate) and experimental repetition. A least significant difference value [LSD (0.05)] was calculated to compare all of the fertilizer treatments. Orthogonal contrasts were used to compare the effects of the different Cu sources in the plant parameters (DM yield and both soluble MES- and EDDS-extractable and total Cu concentrations) and in the soil extractable Cu concentrations.

RESULTS

Spinach Response to Cu Fertilization. The effects of the different fertilizer treatments on plant DM production and Cu concentration in spinach tissues (leaves FM and whole shoots DM) are shown in Table 1.

In all cases, multifactor variance analyses showed significant differences between treatments ($P < 0.0001$). The application of the Cu treatments always produced increases in plant DM production in comparison with the control treatment (untreated soil). The yield of spinach from the control was 2.45 g of shoot DM, compared with an average of 3.98 g of shoot DM for the other treatments. Applying Cu to the soil increased shoot DM, with respect to the control, by a factor of between 1.2 (Cu-ORG for 1 mg Cu kg^{-1}) and 2.0 (Cu-HEDTA for 3 mg Cu kg^{-1}). For most of the Cu sources, the overall effect was that the DM yield increased as the Cu application rate increased. Even so, this parameter was not significantly different for the three different rates applied in the case of the Cu-D-H-E source and the DM yield even decreased for the highest rate applied (3 mg Cu kg^{-1}) in the case of the Cu-EDTA source. Cu-HEDTA, Cu-EDTA, and Cu-D-H-E were the sources that produced the highest levels of plant DM production. Orthogonal contrasts showed that the Cu-HEDTA source produced plant DM yields that were similar to those produced by the Cu-EDTA and Cu-D-H-E fertilizers (see Table 1).

All of the Cu treatments produced significant increases in soluble Cu concentrations (MES- and EDDS-extractable Cu) determined in the FM of young leaves with respect to the control, but the rates of increase varied according to the sources, and only weakly depended on the extraction reagent used (MES or EDDS). Although there was a highly significant ($P < 0.0001$) and positive correlation between EDDS- and MES-extractable Cu, the Cu concentrations extracted with the EDDS reagent were slightly higher than those obtained using the MES reagent. This could have been due to the fact that the EDDS chelating agent is stronger than the MES complexing agent; as a result, EDDS forms more stable chelates with Cu^{2+}. Considering the results of the two extraction methods, the lowest soluble Cu concentrations occurred with the smaller application rates of the inorganic (Cu-OXYCL) and organic complexes (Cu-LS, Cu-GLU, Cu-GA-MGLU, and Cu-ORG) sources. In contrast, the greatest increases were recorded for the highest application rates of the two fertilizers that contained the synthetic chelates Cu-EDTA and/or Cu-DTPA (Cu-EDTA and Cu-D-H-E); for example, applications at 3 mg Cu kg^{-1} increased the MES-extractable Cu concentration in the FM with respect to the control by factors of 4.9 and 4.1 for Cu-EDTA and Cu-D-H-E, respectively.

The total Cu concentration in the DM of whole shoots varied even more than the soluble Cu concentrations in the leaves: from 9.55 mg kg^{-1} DM (control treatment) to 52.51 mg kg^{-1} DM (Cu-EDTA for 3 mg Cu kg^{-1}). This total concentration also increased as the Cu application rate increased and at a similar rate to that of the soluble Cu concentration in the FM of the leaves. The lowest total Cu concentrations in shoot DM occurred with the organic complexes (except Cu-LS) and especially with the inorganic source (Cu-OXYCL). In contrast, the maximum values were also obtained for the Cu-D-H-E source and particularly with the Cu-EDTA fertilizer; for example, applications at 3 mg Cu kg^{-1} increased the total Cu concentration with respect to the control by factors of 5.0 and 5.5, respectively.

Positive and highly significant ($P < 0.0001$) correlations were found between the total Cu concentration in whole shoot DM and both soluble Cu concentrations in leaf FM. Orthogonal contrasts showed that the Cu-EDTA and Cu-D-H-E sources produced the best results taking into account the three plant Cu concentrations studied ($P < 0.00001$) (see Table 1). The other synthetic fertilizer (Cu-HEDTA) produced better results than the organic complexes and inorganic sources considering plant DM production and Cu concentration in leaf FM tissues ($P < 0.001$), but not in relation to total Cu concentration in shoot DM. In relation to the inorganic fertilizer (Cu-OXYCL), this produced higher DM yield ($P < 0.00001$) than the organic complexes (Cu-LS, Cu-GLU, Cu-GA-MGLU, and Cu-ORG) but produced lower total Cu concentration in the DM of whole shoots ($P < 0.00001$). In line with these results, applications of Cu fertilizers to the soil always produced greater total Cu shoot uptakes (the product of the DM production and the total Cu concentration in shoots); the amounts of Cu uptake by spinach plants increased as the Cu application rate increased, with the maximum values being associated with the Cu-EDTA and Cu-D-H-E sources.

Soil Cu Concentration. The Cu-extractable concentrations obtained with the one-step extraction methods, using DTPA-TEA, LMWOAs, and $BaCl_2$ as extractants, are shown in Table 2.

In all cases, multifactor variance analyses showed significant differences between treatments ($P < 0.0001$). Copper extracted by DTPA-TEA and LMWOA reagents (both of which assessed potentially available Cu to plants) ranged from 0.30 to 6.45 mg kg^{-1} and from 0.04 to 1.76 mg kg^{-1}, respectively. According to the DTPA-TEA-extractable Cu concentrations and textural class of the studied soil, there was less available Cu in the control (unfertilized soil) than the level reported as deficient level for many plants (<0.4 mg kg^{-1}).[38] In contrast, for the fertilized soils, the quantities of available Cu would still have been more than sufficient to satisfy the requirements of most of crops, because the lowest concentrations obtained were much higher than the soil deficient level. The Cu concentrations extracted with the LMWOA reagent exhibited similar behavior to DTPA-TEA-extractable Cu, although their levels were considerably lower. Taking into account the results obtained using both extraction methods, the lowest available Cu concentrations occurred with the inorganic and organic complexes sources (except Cu-LS). In contrast, the largest quantities of available Cu for subsequent crops considering both single extraction methods were always associated with the Cu-EDTA and the Cu-D-H-E fertilizers. Copper extracted with $BaCl_2$ (easily leachable Cu) exhibited similar behavior to available Cu; however, the concentrations extracted were significantly lower than those obtained using DTPA-TEA and similar to those obtained using LMWOA reagents (see Table 2). There were significant ($P < 0.002$) and positive correlation between the two Cu concentrations predicted as plant available (DTPA-TEA and LMWOAs). Furthermore, there were also

Table 2. DTPA-TEA-, Mixture of Low-Molecular-Weight Organic Acid (LMWOA)-, and $BaCl_2$-Extractable Cu in Soil with the Application of Different Cu Fertilizer Treatments[a]

treatment	Cu rate (mg kg^{-1})	DTPA-TEA (mg kg^{-1})	LMWOAs (mg kg^{-1})	$BaCl_2$ (mg kg^{-1})
control	0	0.30	0.04	0.10
Cu-OXYCL	1	2.68	0.13	0.10
	2	4.71	0.24	0.13
	3	4.92	0.28	0.22
Cu-LS	1	4.36	0.24	0.13
	2	5.24	0.36	0.16
	3	5.76	0.42	0.17
Cu-GLU	1	2.94	0.13	0.17
	2	4.54	0.20	0.27
	3	5.25	0.28	0.30
Cu-GA-MGLU	1	2.20	0.16	0.10
	2	5.02	0.29	0.23
	3	5.41	0.32	0.33
Cu-ORG	1	2.43	0.13	0.13
	2	3.67	0.19	0.23
	3	5.18	0.27	0.27
Cu-HEDTA	1	2.98	0.24	0.26
	2	4.49	0.43	0.32
	3	5.95	0.71	0.43
Cu-EDTA	1	3.91	0.72	1.07
	2	5.04	1.34	1.73
	3	6.44	1.76	2.17
Cu-D-H-E	1	3.09	0.40	0.73
	2	4.37	0.87	1.33
	3	6.45	1.54	2.10
LSD[b] (0.05)		0.51	0.17	0.16
orthogonal contrasts[c]				
EDTA and D-H-E vs other Cu sources		10.2***	14.88***	23.44***
HEDTA vs OXYCL, LS, GLU, GA-MGLU, and ORG		2.82	3.21***	2.14*
OXYCL vs LS, GLU, GA-MGLU, and ORG		−2.76	−0.39	−0.69

[a] Significant differences between treatments for the three parameters were at $P < 0.0001$. [b] Least significant differences between treatments at $P \leq 0.05$. [c] Contrast values are the difference between treatment means: ***, **, and * denote significance at 0.00001, 0.0001, and 0.001, respectively.

significant and positive correlations with easily leachable Cu, although these correlations showed a much higher degree of significance for the LMWOA ($P < 0.0001$) than for the DTPA-TEA extraction method ($P < 0.03$). Orthogonal contrasts showed that the Cu-EDTA and Cu-D-H-E fertilizers produced the highest quantities of theoretically available Cu for subsequent crops ($P < 0.00001$) but also of larger concentrations of easily leachable Cu ($P < 0.00001$) (see Table 2). The other synthetic fertilizer studied (Cu-HEDTA) performed better than the inorganic and organic complexes sources in terms of providing available Cu for a subsequent crop applying the LMWOA method ($P < 0.00001$), but not when the DTPA-TEA extraction method was used. Moreover, this fertilizer produced higher concentrations of easily leachable Cu than the others ($P < 0.001$). There were no significant differences between the inorganic and organic complexes sources ($P > 0.05$) for any of the three single extraction methods used.

The Cu distribution between fractions for the control and the different fertilizer treatments are shown in Figure 1. The concentrations in the different soil Cu fractions very much depended on the different fertilizer treatments applied; however, the main overall effect was that adding Cu fertilizers to this soil produced increases in the Cu concentration in the three first extracted fractions (WS, EX, and SORB) of the SEP. These increases differed according to the fertilizer and the rate applied. Orthogonal contrasts showed that Cu fertilization with Cu-EDTA and Cu-D-H-E sources produced greater increases in the WS and EX fractions than the others ($P < 0.00001$). For both fertilizers, the increases in Cu concentration in the WS fraction were particularly important: 75, 122, and 150 times greater than in the control soil for Cu-EDTA application rates of 1, 2, and 3 mg kg^{-1}, respectively; and 49, 75, and 140 times greater than in the control soil for Cu-D-H-E application rates of 1, 2, and 3 mg kg^{-1}, respectively. This was consistent with the fact that the Cu-EDTA and Cu-D-H-E sources produce high quantities of both available Cu for subsequent crops and easily leachable Cu (see Table 2).

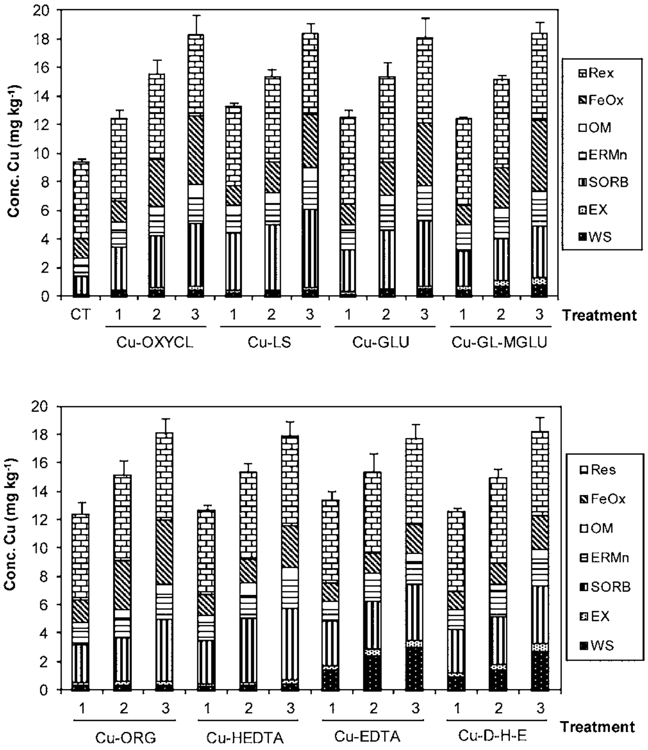

Figure 1. Distribution of Cu fractions in soil at the moment of spinach plants harvest for the control treatment (CT) and the fertilizer treatments with 1, 2, and 3 mg Cu kg^{-1} soil as copper oxychloride (Cu-OXYCL), copper lignosulfonate (Cu-LS), copper gluconate (Cu-GLU), copper galacturonate-monogluconate (Cu-GA-MGLU), copper bis(ethoxydihydroxydiethylamino)sulfate (Cu-ORG), Cu-HEDTA, Cu-EDTA, and Cu-DTPA-HEDTA-EDTA (Cu-D-H-E). Copper fractions in soils are water-soluble (WS), exchangeable (EX), sorbed (SORB), easily reducible (ERMn), organically bound (OM), iron and aluminum oxides bound (FeOx), and residual (RES). The vertical bar at each of the data points represents the standard deviation from the mean of the values.

Orthogonal contrasts showed that the other synthetic source (Cu-HEDTA) produced increases in the WS and EX fractions

Table 3. Simple Correlation Coefficients (R) for Relationships between DTPA-TEA-, Mixture of Low-Molecular-Weight Organic Acid (LMWOA)-, and $BaCl_2$-Extractable Cu and Sequential Extracted Cu Fractions in Soil as well as Cu Uptake and Cu Concentrations in Spinach Tissues (n = 25)[a]

	single extractions			sequential extraction			
	DTPA-TEA	LMWOAs	$BaCl_2$	WS	EXC	SORB	ERMn
Cu uptake[b]	0.696***	0.946†	0.906†	0.905†	0.814†	0.389	0.400*
MES-extractable Cu in FM of leaves	0.660***	0.896†	0.849†	0.857†	0.801†	0.316	0.383
EDDS-extractable Cu in FM of leaves	0.621***	0.856†	0.829†	0.841†	0.851†	0.258	0.395
total Cu in DM of whole shoots	0.640***	0.938†	0.903†	0.899†	0.790†	0.333	0.326

[a] FM, fresh matter; DM, dry matter; Cu fractions in soil are water-soluble (WS), exchangeable (EX), sorbed (SORB), easily reducible (ERMn), organically bound (OM), iron and aluminum oxides bound (FeOx), and residual (RES); MES, morpholino acid; EDDS, ethylenediaminedisuccinic acid. † denotes significance at the 0.0001 level; ***, **, and * denote significance at 0.001, 0.01, and 0.05 levels, respectively. No significant correlations were found between plant parameters and OM, FeOx and RES fractions. [b] Cu uptake (mg) = DM yield (kg) × total Cu (mg kg^{-1} DM).

that were not significantly different from those produced by the inorganic and natural organic sources. In contrast, orthogonal contrasts also showed that the Cu-EDTA and Cu-D-H-E fertilizers produced minor increases in the SORB fraction with respect to the other sources ($P < 0.001$), with Cu-HEDTA and Cu-LS being the sources that exhibited the greatest Cu values in this fraction ($P < 0.00001$).

Comparison of the results of the single extraction methods and SEP for all of the fertilizer treatments reveals that the Cu concentrations in the WS fraction exhibited similar behavior to both estimated available and easily leachable Cu but were smaller than Cu extracted by DTPA-TEA and larger than the Cu concentrations extracted by the LMWOA and $BaCl_2$ reagents. The extractability order of Cu (the mean value of the Cu concentration for all treatments and repetitions) for these different pools of metal was DTPA-TEA (4.29 mg kg^{-1}) > WS (0.80 mg kg^{-1}) > LMWOAs (0.53 mg kg^{-1}) ∼ $BaCl_2$ (0.47 mg kg^{-1}). Furthermore, the values of DTPA-TEA-extractable Cu were of a similar magnitude to the sum of soil-extractable Cu in the first three fractions of the SEP (WS + EX + SORB). The values of LMWOA- and $BaCl_2$-extractable Cu were highly significant ($P < 0.0001$) and positively correlated with the Cu concentrations in the two most active fractions (WS- and EX-extractable Cu). In contrast, the values of DTPA-TEA-extractable Cu were highly significant and positively correlated with the Cu concentrations in the SORB and ERMn fractions ($P < 0.0001$) and exhibited weaker relationships with the Cu concentrations in the first two extracted fractions of the SEP ($P < 0.02$).

Comparison between Soil Tests and Plant Analyses. Simple correlation analyses were performed to define the relationships between plant parameters (DM yield, total Cu shoot uptakes, and Cu spinach tissue concentrations) and extractable soil Cu contents (available Cu, easily leachable Cu, and metal soil fractions). The spinach DM yield did not correlate with most of the extractable soil Cu contents, except in the case of DTPA-TEA-extractable Cu ($P < 0.003$), and only weakly with some of the Cu fractions of the SEP: SORB and ERMn ($P < 0.02$). As shown in Table 3, significant correlations and positive coefficients were observed between Cu uptake by spinach shoots and concentrations of soil-extractable Cu for each of the three single extraction procedures applied: DTPA-TEA extraction ($P < 0.0002$), LMWOAs ($P < 0.0001$), and $BaCl_2$ ($P < 0.0001$). Significant and positive correlations were also observed between Cu uptake and metal concentrations in first two extracted fractions of the SEP (WS and EX) ($P < 0.0001$). All of the Cu concentrations determined in spinach plants (the total in the whole aerial part of the plant DM and both the MES- and EDDS-extractable concentrations in FM leaves) were highly and positively correlated with the concentrations of LMWOA- and $BaCl_2$-extractable Cu ($P < 0.0001$) (see Table 3). Significant correlations were also found between Cu plant concentrations and DTPA-TEA-extractable Cu, but they had a lower degree of significance (P < 0.001−0.0004). Moreover, all of the Cu concentrations determined in spinach tissues were positively correlated with the first two extracted fractions of the SEP ($P < 0.0001$) and especially with the most labile of them (WS). In contrast, no significant correlation was found between plant Cu concentrations and any of the other Cu fractions of the SEP.

DISCUSSION

Spinach Response to Cu Fertilization. The total Cu concentration in spinach DM cultivated in the untreated soil (control) was <10 mg kg^{-1}, which is the concentration considered critical for dried whole shoots for many crops.[42] Applying Cu fertilizers to the soil always increased the Cu concentration in shoot DM, but not significantly with respect to the control for the lowest application rate of the inorganic source (Cu-OXYCL). The highest total uptakes and both soluble and total Cu concentrations of Cu in spinach tissues were associated with the application of Cu-EDTA and Cu-D-H-E sources and therefore significantly improved the nutritional value of the crop with regard to its Cu contribution to the human diet. The total Cu concentrations in shoot DM were >30 mg kg^{-1} for these two Cu fertilizers applied at the two highest rates (2 and 3 mg Cu kg^{-1}); this is within the range of toxic levels reported for some crops in the literature.[43−45] Nevertheless, no visual phytotoxic symptoms were observed in any of the cases for these fertilizer treatments, and spinach DM yields were only slightly reduced in the case of the highest application rate (3 mg Cu kg^{-1}) of the Cu-EDTA source. This was not unexpected because the toxicity level of Cu in plants largely depends on the species, type of culture, and part of the plant tissue sampled. For example, for a lettuce crop, the Cu toxicity level ranged from 10.7 to 13.5 mg Cu kg^{-1} of shoot DM.[46,47] Data on Cu toxicity affecting spinach crops are limited. Using a 0.5 μM Cu nutrient solution, Ouzounidou et al.[48] reported that the Cu concentration in the DM of mature spinach plant leaves (variety Wonderful) was 25 mg Cu kg^{-1} and that this increased to 729 mg Cu kg^{-1} when the Cu concentration in the nutrient solution increased to 160 μM.

One important parameter in the study of the relative efficiency of any fertilizer is the percentage of its use by the crop. The percentage of Cu used by spinach plants (Cu utilization) can be calculated using the following equation:

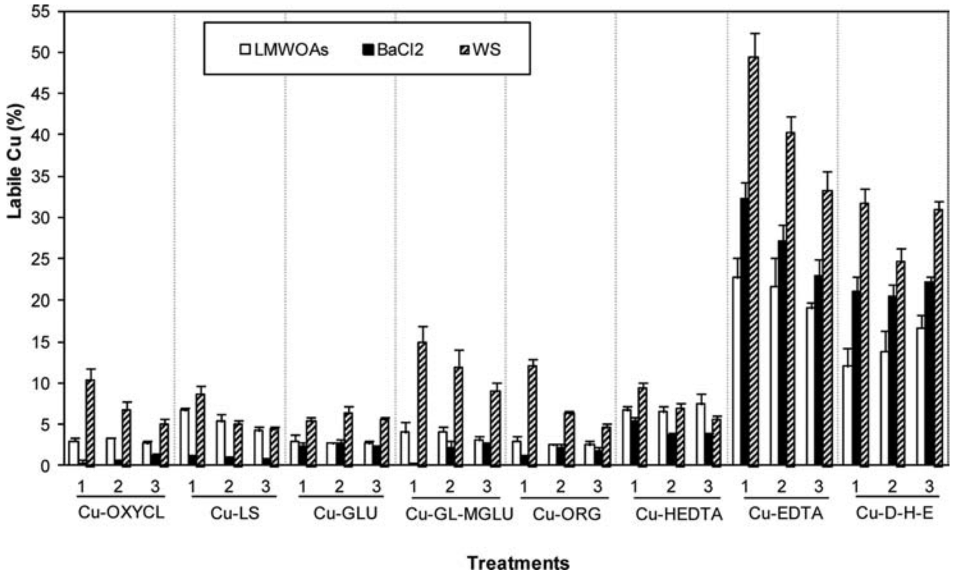

Figure 2. Percentages of labile Cu in soil with respect to applied Cu at the moment of spinach plant harvest with 1, 2, and 3 mg Cu kg^{-1} soil as copper oxychloride (Cu-OXYCL), copper lignosulfonate (Cu-LS), copper gluconate (Cu-GLU), copper galacturonate-monogluconate (Cu-GA-MGLU), copper bis(ethoxydihydroxydiethylamino)sulfate (Cu-ORG), Cu-HEDTA, Cu-EDTA, and Cu-DTPA-HEDTA-EDTA (Cu-D-H-E). The vertical bar at each of the data points represents the standard deviation from the mean of the values.

$$\text{Cu utilization (\%)} = \frac{\text{Cu uptake (treatment)} - \text{Cu uptake (control)}}{\text{Cu applied}} \times 100$$

The highest Cu percentages used by the spinach crop were those involving the low application rate for Cu-EDTA (0.88%) and Cu-D-H-E (0.89%). Copper applied with Cu-EDTA and Cu-D-H-E at higher rates produced lower values of metal use (0.62−0.68%). The other Cu treatments were less effective and particularly those involving Cu-OXYCL and Cu-GLU applied at the two highest rates, which produced values of Cu utilization of less than 0.20%. For a lettuce crop, percentages of Cu utilization ranging from 0.19 to 0.58 were reported by Gonzalez and Alvarez.[47]

Soil Cu Status. Considering the results for all the soil chemical extractions, the largest values of Cu in the most labile forms of the soil (LMWOA-, $BaCl_2$-, and WS-extractable) were always associated with the fertilizers Cu-EDTA and Cu-D-H-E. The percentage of applied Cu remaining in these forms when the spinach was harvested for each fertilizer treatment was calculated according to the following equation:

$$\text{labile Cu (\%)} = \frac{\text{labile Cu (treatment)} - \text{labile Cu (control)}}{\text{Cu applied}} \times 100$$

The values obtained are shown in Figure 2. We observed that when the Cu-EDTA and Cu-D-H-E sources were used (at the three different rates), a high proportion of the applied Cu remained in the soil in a form that was potentially available for subsequent crops; for example, for Cu-EDTA applied at a rate of 1 mg Cu kg^{-1}, about 50% remained in the upper layer of the soil in the pots (0−8 cm) in a WS-extractable pool. In contrast, the percentages of labile Cu associated with the other Cu treatments that were applied were far smaller. This could be mainly explained by the fact that both fertilizers contained Cu chelates of remarkably high stability:[49] Cu-DTPA and/or Cu-EDTA [log $K_{\text{Cu-DTPA}}$ = 22.65, log $K_{\text{Cu-EDTA}}$ = 19.70, with an ionic strength of 0.01 mol L^{-1}].[50] However, Cu forms with the HEDTA ligand a less stable chelate (log $K_{\text{Cu-HEDTA}}$ = 18.25, with an ionic strength of 0.01 mol L^{-1}).[50] Also, Cu forms intermediate stability complexes with naturally occurring organic compounds.[51] Moreover, in our study, the negative charge of applied Cu-chelates − mainly the sources containing EDTA and DTPA chelating agent −- would have reduced Cu sorption. According to McBride,[52] the EDTA chelating agent competes very effectively with clay exchange sites, maintaining virtually all of the added Cu^{2+} in solution as an organic complex. The sources that contained Cu chelated by EDTA and/or DTPA (Cu-EDTA and Cu-D-H-E) may therefore have been able to maintain higher Cu concentrations in the soil solution than the other sources. The greater water Cu extractability in the soils could have resulted from a greater DOC concentration in the soil solution. According to Temminghoff et al.,[53] at pH 6.6 about 99% of the Cu in solution was bound by DOC. In our experiment with a soil with a low organic matter content, which is common in Mediterranean soils, both the WS-Cu and DOC contents in the soil solutions depended on the stability of the Cu chelates applied (e.g., the Cu-EDTA molecule contains 33% of soluble carbon) and also, although to a lesser extent, on the native dissolved organic matter (DOM) in the soil solution.

Even so, all of the fertilized treatments applied to this soil could provide Cu to a subsequent crop because the lower concentrations provided were still greater than the average critical concentrations. These results would also suggest potential Cu transport within the soil profile after Cu fertilization with Cu-EDTA and Cu-D-H-E sources and therefore a greater risk of Cu leaching under conditions of high soil moisture content. Nevertheless, in this experiment (which was conducted with soil moisture content at 60% of the water-holding capacity) we found that the Cu applied did not move below the rooting zone and always remained within the upper 8 cm of the soil in the pots. Below 8 cm, Cu concentrations were similar to those at the beginning of the experiment.

We found a greater water extractability of Cu in soils (WS-Cu) compared with LMWOAs and $BaCl_2$. Various authors[43,54,55] also observed that deionized water extracted more Cu than other extraction solutions. They noted that the main factors that affected Cu extractability were the pH of the extraction solution, its composition, the soil/solution ratio, and the ionic strength. Impelliteri et al.[56] found that for Cu, metal solubility increased with pH, especially above pH 7. In our experiment with a neutral soil (pH 7.09), the largest amount of Cu extracted was observed for the deionized water extractant, which has a higher pH than the LMWOA extractant. Moreover, according to Hass and Fine,[54] an increase in ionic strength has a substantial effect on Cu solubility because this enhances the coagulation and precipitation of soil colloids together with the metals, such as Cu, associated with them. The lower Cu extractability observed in our experiment with the $BaCl_2$ and LMWOA extractants could therefore be partly explained by their greater ionic strengths (e.g., $BaCl_2$ ionic strength = 0.03 M) as opposed to deionized water. The coagulation effects of the $BaCl_2$ extraction solution were also enhanced by the charge of the ion Ba^{2+}.[54]

Comparison between Soil Tests and Plant Analyses. In our experiment, it was possible to predict Cu uptake (mg) by spinach plants with a great degree of accuracy by determining the DTPA-TEA-, LMWOA-, $BaCl_2$-extractable Cu (mg kg^{-1}) or WS-Cu (mg kg^{-1}) in the soil ($P < 0.0002$). The best fit regression models describing plant uptake were

$$\text{Cu uptake} = 0.045 + 0.093 \times \text{LMWOAs Cu}$$

$$(R^2 = 89\%,\ P < 0.0001)$$

$$\text{Cu uptake} = 0.049 + 0.050 \times \text{WS Cu}$$

$$(R^2 = 82\%,\ P < 0.0001)$$

Linear regression models also fitted significantly well between each of the Cu concentrations (mg kg^{-1}) in the spinach tissues (as the dependent variable) and the chemical forms of Cu (mg kg^{-1}) in the soil (as independent variables). They also indicated that Cu concentrations in spinach plants (total in DM and soluble in FM) could be described as a function of labile soil-extractable Cu (LMWOA- or $BaCl_2$-extractable Cu or WS-Cu) (R^2 = 71−88%; $P < 0.001$). The best fit regression models were

$$\text{total Cu} = 12.15 + 21.64 \times \text{LMWOAs Cu}$$

$$(R^2 = 88\%,\ P < 0.0001)$$

$$\text{MES Cu} = 0.97 + 1.51 \times \text{LMWOAs Cu}$$

$$(R^2 = 80\%,\ P < 0.0001)$$

$$\text{EDDS Cu} = 1.36 + 1.57 \times \text{LMWOAs Cu}$$

$$(R^2 = 73\%,\ P < 0.0001)$$

The results obtained therefore showed that the nutritional state of the spinach crop with respect to this microelement depended on the capacity of the Cu fertilizers to maintain the added Cu as very labile forms. In contrast, linear regression equations obtained between each of the Cu concentrations in the plant tissues and DTPA-TEA-extractable Cu showed a lower degree of significance (R^2 = 39−44%; $P < 0.001$−0.0004), demonstrating that the DTPA method was not as good at predicting the availability of Cu to the spinach crop in this type of soil. From the linear regression study, it appears that the soil extractable Cu in some of the sequential extracted fractions had no effect on the Cu concentration in the plants (e.g., SORB, ERMn, and OM), but they did have a positive influence on DTPA-extractable Cu. It could be therefore extrapolated that the DTPA-TEA reagent would extract larger amounts of metal than the spinach is able to take up, overestimating phytoavailability and therefore assessing potential availability rather than immediate availability. Nevertheless, nonaggressive extractions, such as WS, LMWOAs, and $BaCl_2$, could extract only amounts of micronutrient that would tend to represent short-term available pools.

A close relationship was found between both the total Cu concentration in shoot DM and the total Cu uptake by plants and concentration of soluble Cu extracted from young FM leaves (MES- and EDDS-extractable). Moreover, both soluble Cu concentrations were highly correlated with soil Cu concentrations in the most labile soil pools (LMWOA- or $BaCl_2$-extractable Cu and WS-Cu). This would seem to suggest that a good indicator of the Cu nutritional status of spinach crop could be obtained by simply determining the amount of soluble metal in the FM of young leaves (whether with the MES or EDDS reagent). Independent of this result, determining the amount of soluble Cu by either of the two methods proposed is much easier than applying the methods usually employed to determine total or pseudototal metals in plants. Furthermore, these methods do not require the use of hazardous acids such as HNO_3 and HCl.

In conclusion, the application of fertilizers that contained Cu chelated by EDTA and/or DTPA (Cu-EDTA and Cu-D-H-E) significantly improved the nutritional value of the crop with regard to its Cu contribution to the human diet, providing the highest level of agronomic biofortification. Moreover, Cu applied in these forms was associated with the highest values of Cu utilization by the plants grown in this soil. The largest quantities of Cu in the most labile soil forms were also associated with these two Cu sources, with a large proportion of the applied Cu remaining in a potentially available pool for succeeding crops. Considering all of the results obtained with the eight Cu fertilizers studied for this type of soil−crop combination, it would be advisable to use the Cu-EDTA and Cu-D-H-E sources. It would be recommendable to apply the Cu-EDTA fertilizer at rates of 1 and 2 mg Cu kg^{-1} (∼3 and 6 kg Cu ha^{-1}, respectively), because the Cu rate of 3 mg kg^{-1} (∼9 kg Cu ha^{-1}) produced a decrease in both DM yield and the percentage of soil applied Cu that remained in labile forms for the next crop. The stronger correlations obtained between LMWOA-extractable Cu in soil and the Cu concentrations and Cu uptakes by the plants show the suitability of this soil extraction method for predicting Cu available to spinach plants.

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Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS USED

Cu-D-H-E, Cu-DTPA-HEDTA-EDTA; Cu-EDTA, copper ethylenediaminetetraacetate; Cu-GA-MGLU, copper galacturonate-monogluconate; Cu-GLU, copper gluconate; Cu-HEDTA, copper *N*-2-hydroxyethylethylenediaminetriacetate; Cu-LS, copper lignosulfonate; Cu-ORG, copper bis(ethoxydihydroxydiethylamino)sulfate; Cu-OXYCL, copper oxychloride; DM, dry matter; DTPA, diethylenetriaminepentaacetate; EDDS, ethylenediaminedisuccinic acid; ERMn, easily reducible oxides bound; EX, exchangeable; FeOx, iron and aluminum oxides bound; FM, fresh matter; LMWOAs, low-molecular-weight organic acids; MES, morpholino acid; OM, organically bound; RES, residual; SORB, sorbed; TEA, triethanolamine; WS, water-soluble

■ REFERENCES

(1) Yruela, I. Copper in plants acquisition transport and interactions. *Funct. Plant Biol.* **2009**, *36*, 409−430.

(2) White, J. G.; Zasoski, R. J. Mapping soil micronutrients. *Field Crops Res.* **1999**, *60*, 11−26.

(3) Malhi, S. S.; Karamanos, R. E. A review of copper fertilizer management for optimum yield and quality of crops in the Canadian Prairie Provinces. *Can. J. Plant Sci.* **2006**, *86*, 605−619.

(4) White, P. J.; Broadley, M. R. Biofortification of crops with seven mineral elements often lacking in human diets-iron zinc copper calcium magnesium selenium and iodine. *New Phytol.* **2009**, *182*, 49−84.

(5) Kopsell, D. E.; Kopsell, D. A. Copper. In *Handbook of Plant Nutrition*; CRS Press/Taylor and Frances Group: Boca Raton, FL, 2007; pp 293−328.

(6) Zhang, Y. Q.; Shi, R. L.; Rezaul, K. M.; Zhang, F. S.; Zou, C. Q. Iron and zinc concentrations in grain and flour of winter wheat as affected by foliar application. *J. Agric. Food Chem.* **2010**, *58*, 12268−12274.

(7) Wei, Y. Y.; Shohag, M. J. I.; Yang, X. E.; Zhang, Y. B. Effects of foliar iron application on iron concentration in polished rice grain and its bioavailability. *J. Agric. Food Chem.* **2012**, *60*, 11433−11439.

(8) Gupta, U. C.; Kening, W.; Siyuan, L. Micronutrients in soils, crops and livestock. *Earth Sci. Front.* **2008**, *15*, 110−125.

(9) Lefsrud, M. G.; Kopsell, D. A.; Kopsell, D. E. Nitrogen levels influence biomass elemental accumulations and pigment concentrations in spinach. *J. Plant Nutr.* **2007**, *30*, 171−185.

(10) Shi, J.; Xu, J.; Huang, P. Spatial variability and evaluation of status of micronutrients in selected soils around Taihu Lake China. *J. Soils Sed.* **2008**, *8*, 415−423.

(11) Menzies, N. W.; Donn, M. J.; Kopittke, P. M. Evaluation of extractants for estimation of the phytoavailable trace metals in soils. *Environ. Pollut.* **2007**, *145*, 121−130.

(12) Soriano-Disla, J.; Gómez, I.; Navarro-Pedreño, J.; Lag-Brotons, A. Evaluation of single chemical extractants for the prediction of heavy metal uptake by barley in soils amended with polluted sewage sludge. *Plant Soil* **2010**, *327*, 303−314.

(13) Lindsay, W. L.; Norvell, W. A. Development of a DTPA soil test for zinc, iron, manganese, and copper. *Soil Sci. Soc. Am. J.* **1978**, *42*, 421−428.

(14) Feng, M. H.; Shan, X. Q.; Zhang, S. Z.; Wen, B. A comparison of the rhizosphere-based method with DTPA, EDTA, $CaCl_2$ and $NaNO_3$ extraction methods for prediction of bioavailability of metals in soil to barley. *Environ. Pollut.* **2005**, *137*, 231−240.

(15) Feng, M. H.; Shan, X. Q.; Zhang, S. Z.; Wen, B. Comparison of a rhizosphere-based method with other one-step extraction methods for assessing the bioavailability of soil metals to wheat. *Chemosphere* **2005**, *59*, 939−949.

(16) Fang, J.; Wen, B.; Shan, X. Q; Lin, J. M.; Owens, G. Is an adjusted rhizosphere-based method valid for field assessment of metal phytoavailability? Application to non-contaminated soils. *Environ. Pollut.* **2007**, *150*, 209−217.

(17) Vázquez, S.; Moreno, E.; Carpena, R. O. Bioavailability of metals and As from acidied multicontaminated soils. Use of white lupin to validate several extraction methods. *Environ. Geochem. Health* **2008**, *30*, 193−198.

(18) Meers, E.; Samson, R.; Tack, F. M. G.; Ruttens, A.; Vandegehuchte, M.; Vangronsveld, J.; Verloo, M. G. Phytoavailability assessment of heavy metals in soils by single extractions and accumulation by *Phaseolus vulgaris*. *J. Exp. Environ. Bot.* **2007**, *60*, 385−396.

(19) Schultz, E.; Joutti, A.; Räisänen, M.; Lintinen, P.; Martikainen, E.; Lehto, O. Extractability of metals and ecotoxicity of soils from two old wood impregnation sites in Finland. *Sci. Total Environ.* **2004**, *326*, 71−84.

(20) Räisänen, M. L.; Kashulina, G.; Bogatyrev, I. Mobility and retention of heavy metals arsenic and sulfur in podzols at eight locations in northern Finland and Norway and the western half of the Russian Kola Peninsula. *J. Geochem. Explor.* **1997**, *59*, 175−195.

(21) Xiaorong, W.; Mingde, H.; Mingan, S. Copper fertilizer effects on copper distribution and vertical transport in soils. *Geoderma* **2007**, *138*, 213−220.

(22) Shan, X. Q.; Wang, Z. W.; Wang, W. S.; Zhang, S. Z.; Wen, B. Labile rhizosphere soil solution fraction for prediction of bioavailability of heavy metals and rare earth elements to plants. *Anal. Bioanal. Chem.* **2003**, *375*, 400−407.

(23) Marschner, H. In *Mineral Nutrition of Higher Plants*; Academic Press: London, UK, 1986.

(24) Gupta, U. C. Copper in crop and plant nutrition. In *Handbook of Copper Compounds and Applications*; Richardson, H. W., Ed.; Dekker: New York, 1997; pp 203−229.

(25) Reuter, D. J.; Robinson, J. B. In *Plant Analysis: an Interpretation Manual*; CSIRO Publishing: Collingwood, Australia, 1997.

(26) Scaife, A.; Turner, M. In *Diagnosis of Mineral Disorders in Plants − V. 2 − Vegetables*; Robinson, J. B. D., Ed.; H. M. Stationery Office, Agricultural Research Council: London, UK, 1983.

(27) Franzen, D. W.; McMullen, M. V.; Mosset, D. S. Spring wheat and durum yield and disease responses to copper fertilization of mineral soils. *Agron. J.* **2008**, *100*, 371−375.

(28) Goh, T. B.; Karamanos, R. E. Copper fertilizer practices in Manitoba. *Can. J. Plant Sci.* **2006**, *86*, 1139−1152.

(29) Rehm, G. W. Response of hard red spring wheat to copper fertilization. *Commun. Soil Sci. Plant Anal.* **2008**, *39*, 2411−2420.

(30) Iacuzzo, F.; Gottardi, S.; Tomasi, N.; et al. Corn salad (*Valerianella locusta* (L.) Laterr.) growth in a water-saving floating system as affected by iron and sulfate availability. *J. Sci. Food Agric.* **2011**, *91*, 344−354.

(31) Gottardi, S.; Iacuzzo, F.; Tomasi, N.; et al. Beneficial effects of silicon on hydroponically grown corn salad (*Valerianella locusta* (L.) Laterr) plants. *Plant Physiol. Biochem.* **2012**, *56*, 14−23.

(32) Malhi, S. S; Cowell, L.; Kutcher, H. R. Relative effectiveness of various sources methods times and rates of copper fertilizers in improving grain yield of wheat on a Cu-deficient soil. *Can. J. Plant Sci.* **2005**, *85*, 59−65.

(33) Liñan, V. C. In *Vademecum de Productos Fitosanitarios y Nutricionales 2012*; Agrotécnicas: Madrid, Spain, 2012.

(34) Obrador, A.; Novillo, J.; Alvarez, J. M. Mobility and availability to plants of two zinc sources applied to a calcareous soil. *Soil Sci. Soc. Am. J.* **2003**, *67*, 564−572.

(35) Gonzalez, D.; Obrador, A.; Lopez-Valdivia, L. M.; Alvarez, J. M. Effect of zinc source applied to soils on its availability to navy bean. *Soil Sci. Soc. Am. J.* **2008**, *72*, 641−649.

(36) Rodríguez-Lucena, P.; Hernández-Apaolaza, L.; Lucena, J. J. Comparison of iron chelates and complexes supplied as foliar sprays and in nutrient solution to correct iron chlorosis of soybean. *J. Plant Nutr. Soil Sci.* **2009**, *173*, 120−126.

(37) USDA Soil Survey Staff. In *Keys to Soil Taxonomy*, 11th ed.; U.S. Government Printing Office: Washington, DC, 2010.

(38) Karamanos, R. E. *Micronutrients for irrigated production*; http://www1.agric.gov.ab.ca/$department/deptdocs.nsf/all/ind10751 (Dec 2012).

(39) Sparks, D. L. In *Methods of Soil Analysis*; SSSA Book Series 5; American Soceity of Agronomy: Madison, WI, 1996; Part 3: Chemical methods.

(40) Rahimi, A.; Schropp, A. Carbonic anhydrase activity and extractable zinc as indicators of the zinc supply of plants. *Z. Pflanzenernaehr. Bodenkd.* **1984**, *147*, 572−583.

(41) Pietrzak, U.; McPhail, D. C. Copper accumulation distribution and fractionation in vineyard soils of Victoria, Australia. *Geoderma* **2004**, *122*, 151−166.

(42) Jones, J. B. In *Laboratory Guide for Conducting Soil Tests and Plant Analysis*; CRC Press: Boca Raton, FL, 2001.

(43) Michaud, A. M.; Bravin, M. N.; Galleguillos, M.; Hinsinger, P. Copper uptake and phytotoxicity as assessed in situ for durum wheat (*Triticum turgidum* durum L.) cultivated in Cu-contaminated former vineyard soils. *Plant Soil* **2007**, *298*, 99−111.

(44) Mocquot, B.; Vangronsveld, J.; Clijsters, H.; Mench, M. Copper toxicity in young maize (*Zea mays* L.) plants effects on growth mineral and chlorophyll contents and enzyme activities. *Plant Soil* **1996**, *182*, 287−300.

(45) Berenguer, P.; Cela, S.; Santiveri, F.; Boixadera, J.; Lloveras, J. Copper and zinc soil accumulation and plant concentration in irrigated maize fertilized with liquid swine manure. *Agron. J.* **2008**, *100*, 1056−1061.

(46) MacNicol, R. D.; Beckett, P. H. T. Critical tissue concentrations of potentially toxic elements. *Plant Soil* **1985**, *85*, 107−129.

(47) Gonzalez, D.; Alvarez, J. M. Effects of copper chelates on lettuce response, leaching, and soil status. *Soil Sci. Soc. Am. J.* **2013**, *77*, 546−557.

(48) Ouzounidou, G.; Illias, I.; Tranopoulou, H.; Karataglis, S. J. Amelioration of copper toxicity by iron on spinach physiology. *J. Plant Nutr.* **1998**, *21*, 2089−2101.

(49) Aboulroos, S. A. Reaction of EDTA, DTPA and EDDHA complexes of zinc, copper and manganese with a calcareous soil. *Z. Pflanzenernaehr. Bodenkd* **1981**, *144*, 164−173.

(50) Lindsay, W. L. In *Chemical Equilibria in Soils*; Wiley: New York, 1979.

(51) McBride, M. B. Reactions controlling heavy metal solubility in soils. *Adv. Soil Sci.* **1989**, *5*, 1−56.

(52) McBride, M. B. Copper in solid and solution phases of soil. In *Copper in Soils and Plants*; Loneragan, J. F. et al., Eds.; Academic Press: Sydney, Australia, 1981; pp 25−45.

(53) Temminghoff, E. J. M.; Van der Zee, S. E. A. T. M.; De Haan, F. A. M. Copper mobility in a copper-contaminated sandy soil as affected by pH and solid and dissolved organic matter. *Environ. Sci. Technol.* **1997**, *31*, 1109−1115.

(54) Hass, A.; Fine, P. Sequential selective extraction procedures for the study of heavy metals in soils sediments and waste materials − a critical review. *Crit. Rev. Environ. Sci. Technol.* **2010**, *40*, 365−399.

(55) He, Z. L.; Zhang, M.; Yang, X. E.; Stoffella, P. J. Release behaviour of copper and zinc from sandy soils. *Soil Sci. Soc. Am. J.* **2006**, *70*, 1699−1707.

(56) Impelliteri, C. A.; Lu, Y.; Saxe, J. K.; et al. Correlation of the partitioning of dissolved organic matter fractions with the desorption of Cd, Cu, Ni, Pb and Zn from 18 Dutch soils. *Environ. Int.* **2002**, *28*, 401−410.