Factors determining Zn availability and uptake by plants in soils developed under Mediterranean climate

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ABSTRACT

Zinc deficiency is an extended agronomic problem, particularly in staple food crops such as cereals. The availability of Zn to plants is ruled by soil properties, biological factors in the rhizosphere, and interaction with other nutrients. These factors may constrain the predictive value of Zn availability indices. This work aimed at assessing the soil factors that affect the absorption of Zn by plants and improving the predictive value of conventional indices. To this end, an experiment was performed using durum wheat (*Triticum durum* L.) grown on a set of soils developed under Mediterranean climate.

In calcareous soils, Zn uptake by plants decreased with increased clay content and Olsen P (P_{Olsen}), meanwhile in non-calcareous soils it decreased with increased crystalline Fe oxides content. Biological factors such as microbial activity and organic anion exudation in the rhizosphere contribute to Zn uptake by plants. No relationship was found between Zn uptake by plants and the DTPA extractable Zn (Zn_{DTPA}). Pyrophosphate extractable Zn (Zn_{Pyro}) was only related to Zn uptake by plants in calcareous soils ($R^2 = 0.29$; $P < 0.01$). The best estimation of Zn uptake by plants in calcareous soils was obtained with a model involving Zn_{Pyro}/P_{Olsen} ratio and clay content ($R^2 = 0.57$; $P < 0.001$). In non-calcareous soils, Zn uptake by plants was accurately estimated with a model involving Fe bound to crystalline oxides and rhizospheric oxalate (81% of the variance explained). Results reveal the need of discriminating soils according to its carbonate content and the use of soil properties related to Zn adsorption capacity, such as clay and Fe oxide content, and Olsen P for accurate estimation of Zn uptake by plants.

1. Introduction

The deficiency of micronutrients is an extended nutritional imbalance constraining crop production in many agricultural lands in the world (Ryan et al., 2013). Around one-third of cultivated soils have low Zn availability levels for suitable crop production (Broadley et al., 2007; Alloway, 2009; Cakmak and Kutman, 2018). In the Mediterranean basin, Zn deficiency is an extended agronomic problem mostly ascribed to soil chemical properties (e.g. carbonates, pH, low organic matter content) (Rashid and Ryan, 2004; Alloway, 2009). Zn deficiency causes yield decreases, particularly in cereals, which are staple food in many regions of the world (Ryan et al., 2013), frequently without clear deficiency symptoms. Zn deficiency not only implies a productivity constraint, but also a reduction in the grain quality for human consumption, with clear health implications in regions with cereal-based diets (Yang et al., 2007; Cakmak, 2008; Borrill et al., 2014).

The mobility and availability of Zn to plants is ruled by soil properties and biological factors in the rhizosphere (Moreno-Lora et al.,

2019). In this regard, the root and microbial exudates may increase the solubility and mobilization of micronutrients as a result of soil acidification or complexing process (Marschner, 1993; Rengel, 2015). Plants absorb Zn from soil solution as free ions, that may came from different pools: soil solution, soluble organic complexes, and that in equilibrium with the soil solution (easily-desorbable) adsorbed on different minerals (Adhikari and Rattan, 2007; Alloway, 2009; Mousavi, 2011). Nevertheless, the available fraction represents only a small portion of total Zn content in soils, while 80–90% is present in the relatively inactive clay lattice and insoluble precipitated forms (Adhikari and Rattan, 2007; Regmi et al, 2010). Zn concentration in soil solution is very low and its mobility and transport to the root surface is limited by the forms in equilibrium with soil solution (Rengel, 2015).

The supply of Zn from soil to plants is assumed to be governed mainly by its concentration in the parental material, soil chemical properties (pH, organic matter, clay minerals, sesquioxides, carbonates), and nutrient interactions such as that with other micronutrients and the well-known antagonism with P (Adhikari and Rattan, 2007;

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Alloway, 2009; Fageria et al., 2002; Imtiaz et al., 2006; Mousavi, 2011). Sandy soils poor in organic matter are usually poor in total and available Zn, with a limited capacity of replenishing the nutrient absorbed from soil solution (Alloway, 2009; Rashid and Ryan, 2004; Ryan et al., 2013). In calcareous soils, co-precipitation on calcite and chemisorption on Fe oxides are relevant process explaining Zn fixation (Uygur and Rimmer, 2000; Montilla et al., 2003; Buekers et al., 2007; Alloway, 2009; Rengel, 2015; Ryan et al., 2013). Free $CaCO_3$ not only contributes to fix the nutrient by specific and non-specific adsorption onto calcite (Montilla et al., 2003), but also the ensuing high pH reduces its solubility by enhancing the precipitation of Zn hydroxides (Rashid and Ryan, 2004), and by promoting an essentially non-reversible adsorption on Fe oxides (Buekers et al., 2007; 2008).

Fe oxides adsorb Zn through surface complexation in an irreversible process at pH > 6, and by non-specific adsorption which is dependent on pH since negative surface charges increases at increased pH (Buekers et al., 2007; 2008; Stahl and James, 2010; Ryan et al., 2013). The effect of an increased specific surface in Fe oxides, higher in poorly crystalline than in crystalline ones, is a major factor explaining the role of the former in Zn adsorption by surface complexation (Komárek et al. 2018), which increases with reaction time (Shuman, 1977; Buekers et al., 2008; Duffner et al., 2014). In other minerals, such as kaolinite, surface complexation is also deemed to be the dominant mechanism for Zn adsorption (Wang et al. 2017).

It is well known that high P availability levels in soils may negatively affect Zn uptake by plants (Loneragan et al, 1979; Zhang et al., 2012). However, this antagonistic effect seems to vary depending on the different soil minerals that adsorb P and Zn (Sánchez-Rodríguez et al., 2017). Thus, the risk of Zn deficiency induced by P seems to be increased at increased Fe oxides to $CaCO_3$ ratio (Rahmatullah, 2000). Phosphate competition for the adsorption sites may induce Zn deficiency in soils (Ryan et al., 2013). This competition however, is affected by the availability of sorbent sites, which depends on the content of Fe oxides and their crystallinity.

Despite the body of literature published, many aspects of Zn dynamics in soils are not fully understood. Given the relevance of carbonates and Fe oxides in soils, better understanding of their role in Zn reactions and availability is necessary for predicting and managing Zn deficiency in agricultural soils. Despite evidences on the contribution oxides and carbonates to Zn adsorption in soils (Ji and Cang, 1993; Montilla et al., 2003), the role of crystalline and poorly crystalline oxides in basic soils, or the relative contribution of oxides and carbonates to Zn availability remains to some extent not understood. This role of adsorbent surfaces is affected by the interaction between Zn and P since these nutrients are adsorbed on the same minerals, and even may compete for adsorption sites (Zhao and Selim, 2010). On the other hand, the coadsorption of P and Zn has proved crucial explaining Zn retention on poorly crystalline oxides (Liu et al. 2016). Thus, assessment of Zn dynamics should also consider the interaction with P dynamics in soil.

Conventional Zn availability indices for assessing the potential crop response to fertilization are usually based on the use of complexing agents, such as DTPA (Lindsay and Norvell, 1978). However, these methods have sometimes little predictive value (Feng et al., 2005; Maqueda et al., 2015). Interaction with other nutrients and soil properties affecting Zn dynamics may constrain the predictive value of Zn availability indices. As mentioned above, these factors governing the dynamics of Zn, and consequently its availability to plants, are not fully understood. Consequently, for improving the estimates of Zn availability and the prediction of the potential response to Zn fertilizers, further knowledge of soil factors ruling Zn availability to plants is required. It may be hypothesized that availability indices may be improved or corrected in order to increase its predictive value if soil factors affecting Zn availability to plants are taken into account.

On these grounds, the objectives defined for the present work were: (i) to study soil factors affecting Zn uptake by plants in a set of representative soils developed under Mediterranean climate, and (ii) to achieve accurate estimates of Zn uptake by plants by using usual Zn extraction procedures in combination with soil properties.

2. Material and methods

2.1. Soils

Thirty nine soil samples were taken from the surface layer in different locations in Spain. Selected soils encompassed the most representative soils order according to the Soil Taxonomy (Soil Survey Staff, 2014), i.e. Inceptisols, Alfisols, Vertisols, and Mollisols, including calcareous and non-calcareous soils. In each selected location, a square with homogeneous soil of approximately 1000 m^2 was defined. Soil homogeneity was considered in terms of expectable soil taxonomy in the location, color, texture, and structure in the surface horizon in the soil. In the defined area, subsamples of the surface layer (0–20 cm) of the soil were randomly taken in 10–12 sampling points. To this end, in each sampling point (1 m^2) eight soil cores (50 mm diameter) were taken to obtain a subsample, and after that all the subsamples from each sampling point were mixed to obtain a composite sample. Each sample was air-dried, homogenized and sieved to < 4 mm for pot experiment to avoid an excessive destruction of soil structure that may affect crop performance in pots and to < 2 mm for chemical analyses and characterization as described by Recena et al. (2017).

2.2. Soil analysis

Soils were analysed for particle size distribution according to the pipette method as described by Gee and Bauder (1986), organic carbon (SOC) by the oxidation method of Walkley and Black (1934), total $CaCO_3$ equivalent (CCE) by the calcimeter method, cation exchange capacity (CEC) by using 1 M NH_4OAc buffered at pH 7 (Sumner and Miller, 1996), electrical conductivity (EC) in the saturation extract, and pH in water at a soil:extractant ratio of 1:2.5. Clay minerals, with determination of relative proportions of kaolinite, illite, and smectite, were studied by X-Ray diffraction spectrometry (XRD) (Philips PW 1830, PANalytical, Madrid, Spain).

Extraction with DTPA according to Lindsay and Norvell (1978) was used as Zn availability index to plants, performing the extraction with 4 g of soil in 20 mL of extractant (0.005 M diethylene-triaminepentaacetic acid, 0.1 M triethanolamine and 0.01 M $CaCl_2$ at pH 7.3), and shaking at 2.7 s^{-1} during 2 h. After that, suspensions were centrifuged at 1260 g for 15 min, the supernatant was collected and Zn was determined by atomic absorption spectrometry (AAS).

Olsen P (P_{Olsen}) (Olsen et al., 1954) was used as P availability index, by determining molybdate reactive P according to Murphy and Riley (1962) in the supernatant after extraction of 2 g of soil with 40 mL 0.5 M $NaHCO_3$ at pH 8.5, and centrifugation at 1260 g during 10 min.

A proxy estimate of the readily soluble nutrient concentration was done with an extraction with 0.01 M $CaCl_2$ (1:10 w/v). After adding the extractant, the suspension was shaken at 2.5 s^{-1} during 30 min in an end-over-end shaker, and the supernatant collected after centrifugation at 1260 g for 10 min. This supernatant was centrifuged again at 17,900 g during 15 min. In the resulting extract, Zn was determined by ICP-MS (Thermo Elemental X-7; Thermo Fisher, Madrid, Spain).

Zinc bound to the organic matter fraction weakly associated with mineral matrix (Lopez-Sangil and Rovira, 2013) was determined through sodium pyrophosphate extraction following the procedure of Bascomb (1968). To this end, 0.25 g of soil was mixed with 25 mL of 0.1 M Na-pyrophosphate ($Na_4P_2O_7$) at pH 10 and shaken at 4 s^{-1} for 16 h. Then, suspensions were centrifuged at 1260 g for 15 min, and the resulting supernatant was centrifuged again at 17,900 g during 30 min to decant possible colloids. Zinc in the extract was determined by AAS.

Different Fe fractions were determined using the sequential Fe fractionation scheme proposed by De Santiago et al. (2008), in four

steps: (i) 0.1 M NaOH + 1 M NaCl; (ii) 0.27 M sodium citrate + 0.11 M NaHCO$_3$ (CB); (iii) 0.2 M sodium citrate at pH 6 + 0.05 M ascorbate (CA), and (iv) 0.27 M sodium citrate + 0.11 M NaHCO$_3$ + 0.12 M sodium dithionite (CBD). The fraction extracted by CA is assumed to correspond to Fe associated with poorly crystalline oxides (Fe$_{ca}$), and CBD to that associated with crystalline Fe oxides (Fe$_{cbd}$). Fe concentration in the extracts was determined by AAS.

Total Zn concentration in soils was determined by X-ray fluorescence spectrometry (XRF) (X-ray fluorescence spectrometer, PANalytical Axios, Madrid, Spain) using the facilities of the Centro de Investigación Tecnología e Innovación de la Universidad de Sevilla (CITIUS).

2.3. Pot experiment

A pot experiment with three replications per soil sample was performed using durum wheat (*Triticum durum* L., cv Amilcar), which is assumed to be more susceptible to Zn deficiency than bread wheat (*Triticum aestivum* L.) (Alloway, 2009). The experiment was intended to assess Zn uptake by plants in each soil, and it was conducted in a growing chamber under controlled environmental conditions involving: 16 h photoperiod with 200 µmol m^{-2} s^{-1} of light intensity, temperature of 25/18 °C, and relative humidity of 45/60%, day/night. Plants were grown in 100 mL pots (3 cm-diameter, 21 cm-high polypropylene cylinders) containing 100 g of soil and only one plant. Previously, seeds were germinated in a perlite-seedbed irrigated with deionized water and grown until Z1.3 of Zadoks scale (Zadoks et al., 1974) during 15 days. After that, homogeneous plants were transplanted in pots. Pots were irrigated daily with a Hoagland type solution free of Zn at pH 6.5 containing (mmol L^{-1}): MgSO$_4$ (2), Ca(NO$_3$)$_2$ (5), KNO$_3$ (5), KH$_2$PO$_4$ (1), KCl (0.05), H$_3$BO$_3$ (0.024), MnCl$_2$ (0.0023), CuSO$_4$ (0.0005) and H$_2$MoO$_4$ (0.0005). The experiment concluded after 36 days of cultivation when plants were at stage Z9.2 of the Zadoks scale. The total volume of nutrient solution consumed per pot along the cycle was, on average, 0.21 L. Irrigation volume allowed a slight drainage in order to avoid salt accumulation in the soil during cultivation, allowing us to maintain similar concentration of nutrients in the solution of the different studied soils.

2.4. Plant analysis

Shoots and roots were harvested and separated. Roots were separated from soil and washed with deionized water in an ultrasonic bath. Shoots and roots were dried in a forced-air oven at 65 °C for at least 48 h to constant weight and dry matter (DM) in each organ was determined. Aliquots of 0.1 and 0.2 g of dried and milled (< 1 mm) root and shoot, respectively, were mineralized at 550 °C during 8 h in a Mufla furnace. After that, ashes were dissolved in 10 mL of 1 M HCl and heated at 100 °C for 15 min to ensure the full recovery of nutrients. Zinc and P concentrations in the resulting digest were determined by AAS and colorimetrically (Murphy and Riley, 1962), respectively. The plant uptake of each nutrient was calculated as the product of DM and its concentration in each organ, minus the amount of the nutrient in the seed.

2.5. Rhizospheric soil analysis

For soil analysis, only rhizospheric soil was considered, and collected by shaking it off from roots in air (Nazih et al., 2001; Wang et al., 2009). Immediately, soil moist samples were sieved to < 2 mm and homogenized to determine organic anions: After soil extraction with 0.1 M NaOH (1:1) at 4 s^{-1} during 90 min (Radersma and Grierson, 2004; García-López and Delgado, 2016), suspension was centrifuged in two 10 min steps at 1260 g and 15,300 g. Then, the supernatant was acidified to pH 2.5 using 1 M H$_2$SO$_4$ and filtered through a 0.2 µm membrane filter. Samples were separated on an HPLC Varian ProStar

410 instrument furnished with a C18 column (Varian, 250 mm × 34.6 mm, 8 µm particle size), using isocratic elution with 98% of 5 mM H$_2$SO$_4$ at pH 2 plus 2% methanol at 0.8 mL min^{-1} as carrier solution and 20 µl of injected volume. Organic anions were detected at 215 nm using a Varian 486 photo-diode array detector. Individual standard solutions of acetic, oxalic, citric, malic, fumaric and succinic acid, all from Sigma (Barcelona, Spain), were used to identify and quantify compounds in the soil extracts.

A portion of rhizospheric soil was air-dried and stored no more than 48 h, for enzymatic analysis. Dehydrogenase activity was determined following the method of Casida et al. (1964) with some modifications: mixing 2 g of soil, 0.0335 g of CaCO$_3$, 1.75 mL of water and 0.5 mL of 3% 2,3,5-triphenyl tetrazolium chloride (TTC) as enzymatic substrate. The mix was incubated in the dark at 37 °C for 24 h. After that, triphenyl formazan (TPF) produced was sequentially extracted in three times by centrifugation at 1260 g for 10 min using 10 mL of ethanol in each extraction. The color intensity in the extract was measured at 485 nm in order to determine the amount of TPF released. ß-Glucosidase activity was quantified according to Eivazi and Tabatabai (1988), by the measure of the *p*-nitrophenol produced from the soil incubation with 0.05 M 4-nitrophenyl-ß-D-glucopyranoside.

2.6. Statistical analysis

Regression and correlation (Pearson correlation coefficients) analysis was performed using Statgraphics Centurion XVI (Statpoint Technologies, 2013) in order to assess relationships between variables. Multiple regression analysis was performed according to the minimum least square method and we used the Akaike information criterion for the estimation of the optimal model dimension, i.e., the number of independent variables to be included in each regression model (Onofri et al., 2016). With this criterion, we prioritized the simplicity of the model. This simplicity implies to take into account the explained variance of the dependent variable, and the number of independent variables considered in the model, in such a way that it penalizes an increased number of independent variables. In addition, to ensure the maximum accuracy of the model, the Mallows' *Cp*-statistic was calculated (Gilmour, 1996). As assumptions for multiple regressions, all the independent variables included in the model were required to be significant in terms of the *t* statistic at P < 0.05, they were not correlated between them, and they were not linear function of the other variables included in the model, i.e., regressions meet the multicollinearity assumption that was checked by using the variance inflation factor (VIF) that should be always below a value of 5 (Marquardt, 1970). For all the linear regressions, normality according to the Kolmogorov-Smirnov test and homoscedasticity by the scatterplot of residuals were checked.

3. Results

3.1. Soil properties

A wide range in properties was observed in the studied soils (Table 1; Fig S1). Twelve of the soils were non-calcareous, and the average CaCO$_3$ equivalent (CCE) of calcareous soils was 252.5 g kg^{-1} (Table 1). The dominant clay minerals were smectite and illite. The average concentration of Fe in poorly crystalline oxides (Fe$_{ca}$) in the whole set of soils was 1.2 g kg^{-1}, meanwhile that of Fe in crystalline oxides (Fe$_{cbd}$) was 9.5 g kg^{-1} (Table 1 shows averages for calcareous and non-calcareous soils). The Fe$_{ca}$/Fe$_{cbd}$ ratio ranged from 0.01 to 0.53, with an average of 0.15. In non-calcareous soils, this ratio was higher than in calcareous ones (Table 1). Thus, crystalline Fe oxides, as expected in soils from Mediterranean environments, were dominant in the studied soils.

The P availability index (P$_{Olsen}$) ranged widely between soils (Table 2 show averages for calcareous and non-calcareous soils; Fig. S1). The Zn availability index (Zn$_{DTPA}$) varied between 0.11 and

Table 1
General properties of studied soils.

Type of soil		Clay	Silt	Sand	OC	CCE	pH	EC	CEC	Smec	Illit	Kaol	Fe_ca	Fe_cbd	Fe_ca/Fe_cbd
		g kg^{-1}						dS m^{-1}	cmol$_c$ kg^{-1}	%			g kg^{-1}		
Calcareous	Mean	270	130	348	8.2	252	8.35	199	23. 6	37	51	8	0.64	9.06	0.10
	SD	126	56	185	2.6	183	0.25	67	9.6	34	31	4	0.57	5.46	0.10
	Max	700	253	660	13.8	706	8.80	397	44.8	87	95	18	2.22	18.84	0.36
	Min	108	52	50	3.2	32	7.70	108	9.7	0	8	1	0.09	1.47	0.01
Non-calcareous	Mean	190	123	685	8.8	0	7.08	96	17.8	22	43	26	2.33	10.05	0.27
	SD	145	55	153	4.7	0	0.99	51	15.9	36	32	21	0.96	5.76	0.14
	Max	540	212	810	17.1	0	8.80	176	58.6	94	94	63	3.92	25.04	0.53
	Min	62	41	330	4.1	0	5.76	30	7.3	0	4	2	0.98	4.18	0.11

Calcareous n = 27; Non-calcareous n = 12; OC, organic carbon; CCE, calcium carbonate equivalent; EC, electrical conductivity; CEC, cation exchange capacity; Smec, smectite; Illit, illite; Kaol, kaolinite; Fe$_{ca}$, Fe in poorly crystalline oxides; Fe$_{cbd}$, Fe in crystalline oxides; Fe$_{ca}$/Fe$_{cbd}$, ratio of Fe in poorly crystalline to that in crystalline oxides.

0.63 mg kg^{-1}, 33 of the soils showing values below the threshold value for deficiency of 0.5 mg kg^{-1} (not shown). Concentrations of pyrophosphate extractable Zn (Zn$_{Pyro}$) in soil were overall greater than Zn$_{DTPA}$, with an average for the whole set of soils of 1.16 mg kg^{-1}. On the other hand, estimates of the readily soluble Zn (CaCl$_2$ extractable, Zn$_{CaCl2}$) were lower than the availability index, it being much higher in non-calcareous soils than in calcareous ones (Table 2; Fig S1). All these extractable Zn pools account for a minor fraction of total Zn in soil, which ranged from 51 to 128 mg kg^{-1} (Table 2; Fig. S1).

After cultivation, the production of organic anions (oxalate was the only one detected) and enzyme activity ranged widely between soils (Table 2). On average, β-glucosidase activity was higher in calcareous soils than in non-calcareous ones, while differences in the oxalate concentration or the dehydrogenase activity were lower between both types of soils.

3.2. Relationship between Zn extractions and soil properties

In calcareous soils, Zn$_{DTPA}$ and Zn$_{Pyro}$ were positively correlated between them, and both with CaCO$_3$ (Table S1). Zn$_{Pyro}$ was negatively correlated with Fe$_{cbd}$ (which in turns negatively correlated to CaCO$_3$), and Zn$_{CaCl2}$ with clay content. In these soils, Zn$_{DTPA}$ was positively correlated with β-glucosidase and dehydrogenase activity, meanwhile Zn$_{Pyro}$ was only correlated with dehydrogenase activity (Table 3). Both enzymatic activities in the rhizosphere were positively correlated with soil organic C (Table 3). Zn$_{Pyro}$ in calcareous soils increased with dehydrogenase activity (Y = exp[–0.3 + 0.38 lnX]; R^2 = 0.28; P < 0.01).

In non-calcareous soils, Zn$_{DTPA}$ correlated positively to enzymatic activities, meanwhile Zn$_{Pyro}$ was not correlated with enzymatic activities, but correlated positively to oxalate concentration in the rhizosphere (Table 4). Zn$_{CaCl2}$ was negatively correlated with pH, clay content, and P$_{Olsen}$, meanwhile it correlated positively to kaolinite (Table 4). Enzymatic activities correlated between them but only dehydrogenase was significantly correlated with organic matter. Both activities were positively correlated with oxalate in the rhizosphere, and these three variables were positively correlated with clay content in soils (Table 4). The concentration of Zn$_{CaCl2}$ increased with increased Fe$_{ca}$ (Y = 1/[−15.6 + 61.7 X$^{−1}$]; R^2 = 0.72; P < 0.001), meanwhile it was not related to Fe$_{cbd}$. Zn$_{CaCl2}$ decreased with increased ratio of P$_{Olsen}$ to Fe$_{ca}$ + Fe$_{cbd}$ (Y = exp[−0.8 − 0.9 X]; R^2 = 0.54; P < 0.01) in non-calcareous soils.

3.3. Zn uptake by plants as related to extractable Zn and soil properties

The uptake of Zn by plants ranged between 5.3 and 29.5 μg plant^{-1}, with an average value of 12.6, and the range of Zn concentration in shoots and roots was 13–73 and 20–65 mg kg^{-1}, respectively (Table 2 shows average values for calcareous and non-calcareous soils). The relationship between Zn$_{DTPA}$, Zn$_{CaCl2}$, (Fig. 1), and total Zn (not shown) in soil with Zn concentration in plant tissues or total uptake of Zn by plants was not significant. There was a linear relationship between Zn$_{Pyro}$ and the total Zn uptake by plants (P < 0.01), which only explained 26% of the variance in the uptake of the nutrient. However, when the relationship was studied for calcareous and non-calcareous soils separately, it was only significant for the former soils, Zn$_{Pyro}$

Table 2
Zinc and phosphorus extracted by different methods before cultivation, and organic anions, enzyme activities, Zn uptake and Zn concentration in roots and shoots after cultivation.

Type of soil		Olsen P	Zn$_{DTPA}$	Zn$_{Pyro}$	Zn$_{CaCl_2}$	XRF Zn	Oxalate	Enzyme activities		Zn uptake	Zn concentration	
								β-GLU	DHA		Root	Shoot
		mg kg^{-1}					mmol kg^{-1}	mg kg^{-1}h^{-1}		μg plant^{-1}	mg kg^{-1}	
Calcareous	Mean	14	0.34	1.15	0.10	73.20	49.80	145.87	2.70	12.13	31.66	31.52
	SD	6	0.16	0.48	0.05	14.27	14.47	62.81	1.30	5.61	9.63	15.30
	Max	28	0.63	2.49	0.24	128.40	78.10	286.27	5.68	29.47	64.70	72.81
	Min	5	0.11	0.43	0.02	60.80	12.29	65.25	0.66	5.26	19.71	13.36
Non-calcareous	Mean	15	0.40	1.22	0.21	65.69	44.34	110.79	2.56	13.75	29.27	30.18
	SD	8	0.07	0.35	0.23	17.67	21.44	75.31	2.29	5.31	5.05	9.74
	Max	28	0.50	1.76	0.70	115.70	92.84	250.02	8.26	22.60	36.69	42.11
	Min	4	0.30	0.46	0.02	50.80	11.82	22.45	0.53	5.83	23.60	13.43

Calcareous n = 27; Non-calcareous n = 12; DTPA, diethylene-triaminepentaacetic acid; Pyro, pyrophosphate; XRF Zn, total Zn in soil determined by X-ray florescence; the only organic anion found at detectable concentration in the rhizosphere was oxalate; β-GLU, β-glucosidase activity; DHA, dehydrogenase activity; Zn uptake, total uptake of Zn by plants, calculated as the product of DM in each organ and its concentration of the nutrient, minus the amount of nutrient present in the seed; β-GLU as the amount of p-nitrophenol released, and DHA activity measured as the amount of triphenyl-formazan produced

Table 3
Correlation coefficients for total Zn uptake and soil properties for calcareous soils.

	Zn_{Pyro}	Zn_{CaCl2}	OC	Clay	pH	CCE	Kaol	Fe_{ca}	Fe_{cbd}	P_{Olsen}	β-GLU	DHA	Oxalate
Zn_{DTPA}	0.69***	0.12	0.37	0.05	0.13	0.43*	−0.02	0.16	−0.28	0.21	0.49*	0.44*	−0.15
Zn_{Pyro}		0.27	0.16	−0.26	0.28	0.69***	0.04	−0.33	−0.42*	0.34	0.29	0.44*	0.06
Zn_{CaCl2}			0.08	−0.53**	0.23	0.19	−0.17	−0.23	−0.21	0.24	0.14	0.20	−0.22
OC				−0.1	−0.05	0.1	0.28	0.02	−0.27	−0.02	0.66***	0.47*	−0.30
Clay					−0.35	−0.03	−0.15	0.39*	0.05	−0.43*	0.22	−0.20	−0.03
pH						0.22	0.3	−0.44*	−0.05	−0.24	−0.12	−0.05	0.12
CCE							−0.17	−0.57**	−0.66***	0.24	0.34	0.25	0.35
Kaol								−0.15	0.24	−0.07	0	0.06	−0.29
Fe_{ca}									0.32	−0.19	−0.01	0.05	−0.30
Fe_{cbd}										−0.16	−0.47*	−0.09	−0.36
P_{Olsen}											−0.18	0.06	0.11
β-GLU												0.63***	−0.14
DHA													−0.26

Zn uptake, total uptake of Zn by plants, calculated as the product of DM in each organ and its concentration of the nutrient, minus the amount of nutrient present in the seed (µg plant^{-1}); DTPA, diethylene-triaminepentaacetic acid; Pyro, pyrophosphate; OC, organic carbon; CCE, calcium carbonate equivalent; Kaol, kaolinite; Fe_{ca}, Fe in poorly crystalline oxides; Fe_{cbd}, Fe in crystalline oxides; β-GLU, β-glucosidase activity; DHA, dehydrogenase activity.
n = 27; *,**,***, significant at P < 0.05, 0.01, and 0.001, respectively

Table 4
Correlation coefficients for total Zn uptake and soil properties for non-calcareous soils.

	Zn_{Pyro}	Zn_{CaCl_2}	OC	Clay	pH	Kaol	Fe_{ca}	Fe_{cbd}	P_{Olsen}	β-GLU	DHA	Oxalate
Zn_{DTPA}	0.58*	−0.15	0.49	0.42	0.00	0.01	0.16	0.33	−0.07	0.59*	0.60*	0.36
Zn_{Pyro}		0.11	0.54	0.24	−0.45	0.07	0.46	0.00	−0.27	0.50	0.51	0.64*
Zn_{CaCl_2}			−0.02	−0.22	−0.76**	0.74**	0.46	0.08	−0.75**	−0.43	−0.31	0.52
OC				0.33	−0.08	0.25	0.57	0.37	−0.36	0.49	0.81**	0.52
Clay					0.49	−0.05	0.53	0.81**	0.16	0.74**	0.76**	0.76**
pH						−0.48	−0.19	0.36	0.80**	0.36	0.32	0.32
Kaol							0.41	0.4	−0.57	−0.23	0.01	−0.03
Fe_{ca}								0.49	−0.41	0.23	0.47	0.56
Fe_{cbd}									−0.06	0.47	0.68*	0.52
P_{olsen}										0.23	0.07	−0.13
β-GLU											0.84***	0.65*
DHA												0.82**

Zn uptake, total uptake of Zn by plants, calculated as the product of DM in each organ and its concentration of the nutrient, minus the amount of nutrient present in the seed (µg plant^{-1}); DTPA, diethylene-triaminepentaacetic acid; Pyro, pyrophosphate; OC, organic carbon; Kaol, kaolinite; Fe_{ca}, Fe in poorly crystalline oxides; Fe_{cbd}, Fe in crystalline oxides; β-GLU, β-glucosidase activity; DHA, dehydrogenase activity.
n = 12; *,**,***, significant at P < 0.05, 0.01, and 0.001, respectively.

explaining 29% of the variance in the Zn uptake (Fig. 1). An improvement of the prediction of Zn uptake in calcareous soils was possible considering also the P availability index (P_{Olsen}); the Zn_{Pyro}/P_{Olsen} ratio explained 40% and 54% of the variance in total Zn uptake and Zn concentration in shoots, respectively (Fig. 2). Nevertheless, this ratio did not have any predictive value for Zn nutrition in plants in non-calcareous soils (Fig. 2). In calcareous soils, the Zn uptake decreased with increased clay content (ln Y = 4.9 − 0.46 ln X; R^2 = 0.24, P < 0.01). The best estimate of Zn uptake in calcareous soils was achieved with the multilinear model involving Zn_{Pyro}/P_{Olsen} and clay content (R^2 = 0.57; P < 0.001; one outlayer excluded) (Fig. 2).

In non-calcareous soils, Zn uptake increased with decreased Fe_{cbd} (Fig. 3), and was not related to Fe_{ca}. In these soils, the best estimate of Zn uptake by plants was achieved with a multilinear model involving Fe_{cbd} and oxalate concentration in the rhizosphere, which explained 81% of the variance in Zn uptake (Fig. 3).

Regarding biochemical properties, in calcareous soils, Zn uptake by plants increased linearly with increased dehydrogenase activity (R^2 = 0.33) and decreased with increased ratio of β-glucosidase to dehydrogenase activity (R^2 = 0.26) (in both cases, P < 0.01, one outlayer excluded; data not shown). Enzymatic activities in non-calcareous soils were not related to Zn uptake (not shown).

In the whole soil dataset, Zn_{Pyro} and clay explained 36% of the variation in Zn uptake by plants (Fig. 4).

4. Discussion

4.1. Soil factors affecting Zn uptake by plants

The relationships between soil properties and Zn uptake by plants and the relationship of the different Zn extractions and Zn uptake differed between calcareous and non-calcareous soils. Thus, we considered more suitable an independent analysis of soil properties affecting Zn uptake in each type of soils.

4.1.1. Calcareous soils

Between the different Zn extractions, only Zn_{Pyro} had some predictive value of Zn uptake by plants in calcareous soils. This allows one to suppose that Zn bound to organic matter plays a key role in Zn availability to plants. In calcareous soils, it is assumed that Zn bound to organic matter may be released to the soil solution more easily than that bound to carbonates (Rashid and Ryan, 2004). However, Zn_{Pyro} was also positively correlated with $CaCO_3$ in these soils (Table S1). Thus, it is unclear if this extraction really reflects Zn bound to organic matter, to carbonates, or both since the pyrophosphate extraction not only disperses organic matter but also may disperse other colloidal particles (De Santiago and Delgado, 2006).

Zn uptake decreased with increased clay content in calcareous soils (Fig. 2). Clay minerals have been described as the main Zn sorbent surfaces in soils (González-Costa et al., 2017). It is expectable specific Zn adsorption on the hydroxilated borders of clay minerals (Wang et al.,
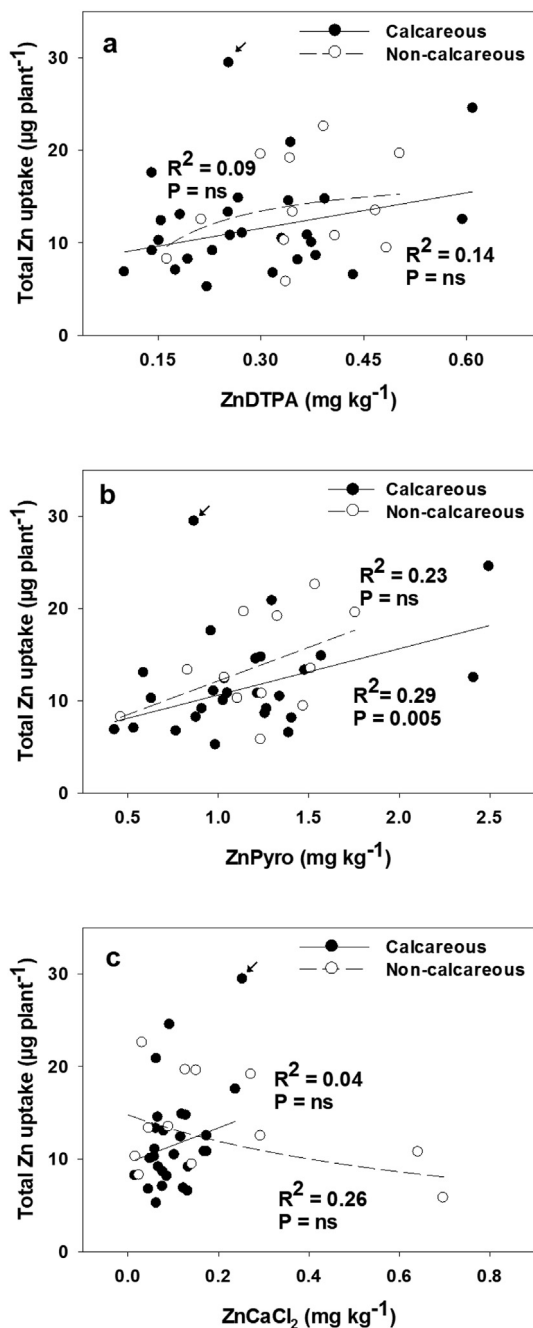
Fig. 1. Relationships between the total Zn uptake by plants and: DTPA extractable Zn (Zn_{DTPA}) (a), Pyrophosphate extractable Zn (Zn_{Pyro}) (b), and $CaCl_2$ extractable Zn (Zn_{CaCl2}) as proxy of the readily soluble Zn (c); linear regression was only significant for the relationship between Zn uptake and Zn_{Pyro} was only significant in calcareous soils ($Y = 5.6 + 5 X$). For the whole set of soils, the relationship was also significant: ($Y = 5.6 + 5.6 X$; $R^2 = 0.26$; $P < 0.01$). Data marked with an arrow was not included in the regression since it was considered an outlayer.

Fig. 2. Relationships of the pyrophosphate extractable Zn (Zn_{Pyro}) to P_{Olsen} ratio and: the total Zn uptake by plants (a), and the Zn concentration in shoots (b) in calcareous soils. Relationships were only significant for calcareous soils: $Y = 7.7 + 374 X^2$ for Zn uptake, and $Y = 17.2 + 1273 X^2$ for Zn concentration in shoots. Data marked with an arrow was not included in the regression since it was considered an outlayer; (c) estimate of the Zn uptake by plants in calcareous soils using the model: Estimated Zn uptake $= 9.9 + 71$ $Zn_{Pyro}/P_{Olsen} - 0.02$ Clay; outlayer marked with an arrow excluded.

2017). This adsorption of Zn in clay is considered almost irreversible at basic pH such as that in calcareous soils (Zhao and Selim, 2010). This agrees with Zn_{CaCl2} being negatively correlated with clay content, since this may reveal that a higher adsorption capacity leads to less readily soluble Zn. The irreversible adsorption of Zn on clay may explain the decreased Zn uptake in the more clayish calcareous soils.

The uptake of Zn by plants was negatively affected, according to the models for estimating it, by P_{Olsen} in calcareous soils. This may be ascribed to the antagonistic effect between these nutrients. In addition,

the simultaneous adsorption of P and Zn has proved crucial explaining Zn retention on Fe oxides (Liu et al. 2016). Perhaps this may contribute to the negative effect of P in calcareous soils. However, there were not evidences of the effect of Fe oxides on the availability of Zn in the studied calcareous soils, and consequently it is difficult to assess the potential contribution of Zn and P coadsorption on Fe oxides to Zn uptake by plants. All this reveals that soil P availability should be taken into account for accurate estimate of Zn availability to plants in calcareous soils.

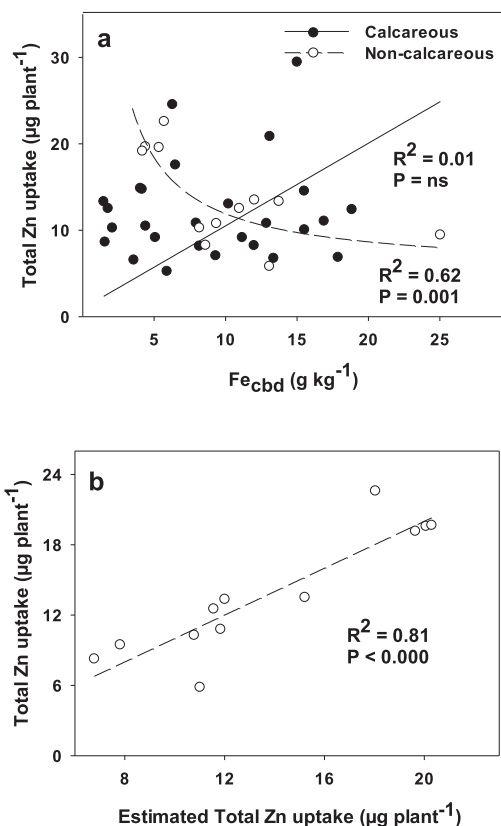In calcareous soils, there are evidences of the contribution of

Fig. 3. (a) Relationship between total Zn uptake by plants and Fe extracted with citrate-bicarbonate-dithionite (Fe_{cbd}) in non-calcareous soils ($Y = 5.4 + 65.2\ X^{-1}$); (b) estimate of the Zn uptake by plants in non-calcareous soils using the model: Estimated Zn uptake $= -3.2 - Fe_{cbd} + 15.6\ \log(\text{Oxalate})$.
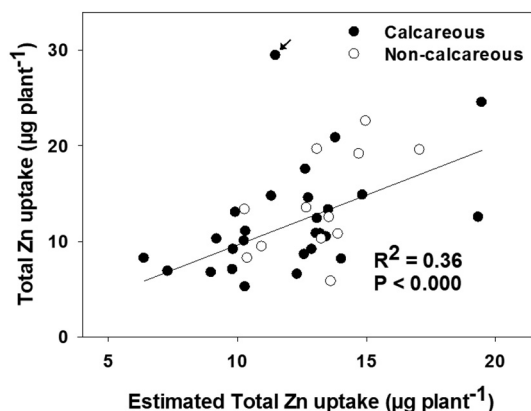

Fig. 4. Estimation of the total Zn uptake by plants in the whole set of soils using the model: Estimated Zn uptake $= 9 + 5\ Zn_{Pyro} - 0.01$ Clay; data marked with an arrow was not included in the regression since it was considered an outlayer.

microbial activity to Zn uptake. This uptake increased with increased dehydrogenase activity in the rhizosphere. However, this may be explained by the correlation between this enzymatic activity and Zn_{Pyro} (Table 3). Perhaps, this latter correlation reveals the contribution of microbial activity (Zn in microbial biomass or complexed by microbial exudates) to the pyrophosphate extractable Zn fraction. In addition, Zn uptake by plants increased with increased ratios of β-glucosidase to dehydrogenase activity after crop harvest. β-glucosidase is deemed a general biological activity indicator which is retained in soil meanwhile dehydrogenase is an intracellular enzyme (Moreno et al., 2015). Thus,

this relationship perhaps reveals that not only microbial activity but also the profile of microbial communities may affect Zn uptake by plants at least in calcareous soils.

4.1.2. Non-calcareous soils

In non-calcareous soils, the role of Zn theoretically bound to organic matter (Zn_{Pyro}) and the antagonistic effect of P was not evident. Surprisingly, in these soils, Zn uptake by plants was not related to any Zn extraction in soils, and it decreased with increased Fe_{cbd}. This reveals that, in non-calcareous soils, the adsorption of Zn on crystalline Fe oxides seems crucial explaining Zn availability to plants. A minimal part of adsorbed Zn may be considered desorbable at neutral and basic pH such as that in our soils (Buekers et al., 2007; Donner et al., 2012). This explains the negative contribution of Fe oxides to Zn uptake in non-calcareous soils.

Poorly crystalline Fe oxides have higher specific surface and greater sorption capacity than crystalline ones. In addition, it is assumed a higher affinity of Zn for poorly crystalline Fe oxides than for crystalline oxides (Bruton et al., 2005). However, only crystalline Fe oxides affected significantly the uptake of Zn by plants, perhaps because they are the most abundant oxides in our soils. Its negative contribution to Zn uptake may reveal a less reversible adsorption on these oxides. Slow adsorption reactions on oxides contribute to explain the lessened Zn availability; however, these slow reactions are unrelated to Fe oxides crystallinity (Buekers et al. 2008). On the other hand, Zn_{CaCl2} increased with increased Fe_{ca}. Thus, it seems that the readily soluble Zn pool is related to poorly crystalline Fe oxides, but not with crystalline Fe oxides. Although Zn_{CaCl2} was unrelated to Zn uptake, its correlation with Fe_{ca} supports the idea of Zn being less strongly bound to poorly crystalline than to crystalline Fe oxides in our non-calcareous soils.

Phosphorus did not have a negative effect on Zn uptake by plants in non-calcareous soils. As mentioned above, coadsorption of P and Zn is a mechanism explaining the retention of Zn on Fe oxides. This agrees with the decreased readily soluble Zn with increased oxide saturation by P as estimated by the ratio of P_{Olsen} to $Fe_{ca} + Fe_{cbd}$. It is assumed that a significant portion of adsorbed Zn became not desorbable and thus not available to plants at neutral and basic pH (Donner et al. 2012). However, the different adsorption mechanism proposed for the co-adsorption of Zn and P on Fe oxides, i.e. ternary complexes with P instead Zn bound to the surface directly (Liu et al. 2016), may affect Zn desorption patterns and consequently its potential availability to plants. This may contribute to explain the lack of negative effect of Olsen P on the Zn uptake by plants in contrast with calcareous soils where the role of Fe oxides on Zn uptake was not evident. However, this result contradicts previous evidences by Sacristán et al. (2018) who described the co-adsorption of P and Zn on Fe oxides as one of the processes in soil explaining P induced Zn deficiency.

Oxalate contributed positively to Zn uptake by plants in non-calcareous soils (Fig. 3). Oxalate may compete for sorption sites and also contributes to dissolve poorly crystalline Fe oxides (De Santiago and Delgado, 2006). This enhances the release of Zn adsorbed on oxides and other sorbent surfaces. Organic anions are released by microorganisms and plant roots under conditions of nutrient deficiency, and oxalate has been described as the main anion released by roots (García-López and Delgado, 2016). Oxalate in rhizosphere may be ascribed at least in part to microbial activity as revealed by the relationship between its concentration and enzyme activity ascribed to microorganisms (β-glucosidase and dehydrogenase; Table 4) observed in non-calcareous soils. This relationship was not observed in calcareous soils, perhaps due to the precipitation of Ca oxalate which is not recovered with the NaOH extraction.

4.2. Estimation of Zn uptake by plants

The commonly used Zn_{DTPA} has no predictive value of Zn uptake in the studied soils (Fig. 1). This agrees with previous works showing the

lack of correlation between this soil test and the Zn uptake, especially in Zn-deficient soils (Wang et al., 2004; Feng et al., 2005). The proxy estimate of readily soluble Zn, i.e. Zn_{CaCl2}, did not relate to Zn uptake by crop. When Zn_{Pyro} and clay were considered in the model, only 36% of the variance in Zn uptake was explained in the whole soil set (Fig. 4), which does not mean a high predictive value of the model.

When soils were separated in calcareous and non-calcareous, Zn uptake estimates were more accurate using single chemical extractions, but only in calcareous soils. In this regard, it should be highlighted that Zn_{Pyro} was the only extraction related to Zn uptake in calcareous soils. Thus, Zn_{Pyro} has higher predictive value if soils are discriminated by carbonate content. In calcareous soils, the Zn_{Pyro}/P_{Olsen} ratio was positively related with Zn concentration in shoots and total uptake, and improved the estimation of Zn uptake (Fig. 2). This reveals the need of considering P availability status in calcareous soils for accurate estimation of Zn uptake by plants in agreement with Sacristán et al. (2018). When besides Zn_{Pyro} and P_{Olsen}, clay content was considered in the predictive model, 57% of the variance in Zn uptake was explained. This means that accurate estimates of Zn uptake can be achieved when considering P availability and soil properties related to Zn adsorption in soils.

In non-calcareous soils, Zn uptake by plants was not related to any Zn extraction. In this case, Zn uptake depended on the main sorbent surfaces in these soils (Fe oxides) and on biological factors in the rhizosphere related to mobilization of Zn from the soil solid phase (oxalate concentration). The effect of organic anions dissolving some Fe oxides may contribute to explain the lack of correlation between Zn uptake by plants and Zn extracted by complexing or dispersive agents (i.e. Zn_{DTPA} and Zn_{Pyro}). It should be remarked that the estimation of Zn uptake is really accurate with a model involving Fe_{cbd} and oxalate in the rhizosphere (81% of the variance explained), but it is not an easy method for practical assessment and further work for more accurate estimation methods is required.

Zn_{DTPA} and Zn_{Pyro} in calcareous soils were correlated with $CaCO_3$. Carbonate bound Zn is assumed to be poorly phytoavailable (Montilla et al., 2003; Chen et al., 2017). Thus, these both extractants are likely releasing non-available Zn forms bound to carbonate between other Zn forms. Perhaps this explains the reduced accuracy of both extractants, in particular DTPA, in predicting Zn uptake by plants in calcareous soils. Alternative extractions based on the use of acids, such as Mehlich, are expected to release carbonate bound Zn as well. Thus, it can be supposed also a poor performance in predicting Zn uptake by crops in calcareous soils. In non-calcareous soils, Zn_{DTPA} and Zn_{Pyro} were correlated between them, but not with other soil components. Thus, the adscription of these extractions to particular Zn pools remains unclear in these soils. On the other hand, positive correlations between Zn_{DTPA} and Zn_{Pyro} with enzymatic activities ascribed to microbial activity were observed in both types of soils (Tables 3 and 4). This may be ascribed to the effect of microbial activity on Zn dynamics and pools in soils, perhaps through the effects of microbial exudations.

The readily soluble Zn (Zn_{CaCl2}) includes that in solution and likely a fraction of that non-specifically adsorbed since, at the pH of these soils, Zn adsorbed by other mechanisms (surface complexation and coprecipitation on oxides and carbonates) cannot be considered readily soluble. However, biological mobilization mechanisms such as organic anion exudation by roots and microorganisms may release Zn bound to oxides and carbonates. In addition, Zn bound to organic matter also contribute to Zn uptake by plants. All this explains the poor predictive value of Zn_{CaCl2} to estimate Zn uptake. Biological mobilization mechanisms likely contribute to explain the poor accuracy of all the Zn extraction methods since these mechanisms makes Zn uptake not dependent on a specific chemical fraction of Zn in soils. This is particularly evident in non-calcareous soils.

5. Conclusions

The usual Zn availability index (DTPA extraction) was not related with the Zn uptake by plants, so that it had little value to predict Zn deficiency. In calcareous soils, the Zn_{Pyro} index explained about 30% of the total Zn uptake. This does not necessarily reveal the relevance of organic bound Zn in explaining its availability to plants since the Zn_{Pyro} was related to soil organic C and carbonates. The estimation of Zn uptake by plants in calcareous soils improved when soil P available and clay were taken into account (57% of the variance explained). In non-calcareous soils, Zn availability indices did not have any predictive value of Zn uptake by plants, and the availability of Zn was ruled by Fe oxides, increasing Zn uptake with decreased concentration of crystalline Fe oxides. Biological factors affecting nutrient mobilization (microbial activity and organic anion exudation) in the rhizosphere contribute to Zn uptake by plants. Results reveal the need of discriminating soils according to its carbonate content and the use of soil properties such as clay content, Fe oxides, and P_{Olsen} for accurate estimation of Zn uptake by plants.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.geoderma.2020.114509.

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