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Effect of Zn binding to phytate and humic substances on its uptake by wheat (*Triticum durum* L.) as affected by carbonates and Fe oxides

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ABSTRACT

Although complexation with soil organic matter may improve zinc (Zn) bioavailability to plants, the effect of Zn sorbent surface on the use of complexed Zn by plants remains unknown. The objective of this research was to elucidate how Zn complexation with humic substances (HS) and phytate affects the uptake of Zn by wheat plants depending on the main sorbent surface in growth media, *i.e.*, carbonates and Fe oxides. To this end, two pot experiments were performed, one using Fe oxide-coated siliceous as the siliceous growth medium sand and the other using a mixture of calcareous sand and siliceous sand as the calcareous growth medium. Each experiment involved three Zn sources, Zn-HS complex, Zn phytate, and $ZnSO_4$. All sources were applied with surface irrigation at two Zn rates (0.25 and 2 mg kg^{-1} growth medium). The Zn-HS complex significantly increased Zn uptake by plants in both media, relative to the other two Zn sources, but no significant difference was observed between Zn phytate and $ZnSO_4$. In the calcareous medium, Zn-HS complex and Zn phytate resulted in significantly higher dry biomass yields of wheat than $ZnSO_4$. In the siliceous medium, spike and shoot dry biomass yields with Zn-HS complex at the low rate and Zn phytate at both rates were not significantly different from those with $ZnSO_4$ at the high rate. After harvest, approximately 50% of the Zn applied as Zn-HS complex remained extractable by diethylenetriaminepentaacetic acid (DTPA), while this proportion was less than 20% for the other Zn sources. Thus, Zn-HS complex and Zn phytate are sources of available Zn for plants, and they are more effective than $ZnSO_4$ in increasing plant growth, particularly when carbonates are the main Zn sorbent surface.

Key Words: calcareous growth medium, micronutrient, organic matter, organic phosphorus, Zn availability, Zn deficiency, Zn sorbent surface, Zn source

INTRODUCTION

Zinc (Zn) is an essential plant nutrient, and its deficiency affects not only crop growth and yield, but also the quality of crops for human consumption. As one of the most common micronutrient disorders in crops, Zn deficiency is common in high-pH soils with low Zn concentrations (Lu *et al*., 2012; Ryan *et al*., 2013; Moreno-Lora *et al*., 2019; Sacristán *et al*., 2019). This is of concern because alkaline calcareous soils make up a third of global agricultural land (Nazir *et al*., 2016; Moreno-Lora and Delgado, 2020). More than three billion people suffer from Zn deficiency worldwide (Cakmak, 2008; Alloway, 2009; White and Broadley, 2011; Clemens, 2014). This is attributed to diets reliant on crops produced in areas with low Zn phytoavailability or with low Zn concentrations, and can be compounded by insufficient fish or animal intake (Graham *et al*., 2007; White and Broadley, 2009; Gibson, 2012; McBeath and McLaughlin, 2014).

Overcoming Zn deficiency in crops requires the use of mineral Zn fertilizers (Rengel and Graham, 1995; Cakmak *et al*., 2010b). Zinc sulfate ($ZnSO_4$) is one of the most commonly used Zn fertilizers because of its high solubility and low cost (McBeath and McLaughlin, 2014; Liu *et al*., 2019). However, mineral fertilizers are inefficient at supplying Zn to crops, particularly in calcareous soils, due to Zn-fixation reactions (Srivastava *et al*., 2009; Lu *et al*., 2012; Zhao *et al*., 2016).

Precipitation, complexation by organic matter, and adsorption on minerals are the main reactions affecting Zn retention and availability to plants in soils because these processes regulate the Zn equilibrium between the solid and solution phases (Moreno-Lora and Delgado, 2020; Recena *et al*., 2021). Zinc adsorption is governed by pH, carbonates, organic matter, clay content and mineralogy, iron (Fe) and aluminum (Al) oxides, and interactions with other nutrients (particularly other metals and phosphates) (Donner *et al*., 2012; Liu *et al*., 2016; Wang *et al*., 2017; Komárek *et al*., 2018; Peng *et al*., 2018). The decreased bioavailability of Zn in alkaline calcareous soils is attributed to the precipitation of $Zn(OH)_2$ or $ZnCO_3$ (Saeed and Fox, 1977; Shukla and Mittal, 1979; Uygu and Rimmer, 2000; Rehman *et al*., 2018), as well as specific and non-specific adsorption onto calcite (Saleh *et al*., 1998; Montilla *et al*., 2003; Alloway, 2009)

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and other soil components such as oxides (Buekers *et al.*, 2007, 2008).

Iron oxides play a crucial role in Zn adsorption in soils, affecting Zn bioavailability (Buekers *et al.*, 2007; Liu *et al.*, 2016). Such adsorption depends on the concentration and crystallinity of Fe oxides (Komárek *et al.*, 2018; Moreno-Lora and Delgado, 2020) and increases with increasing reaction time (slow reactions) and pH (Tye *et al.*, 2003; Buekers *et al.*, 2008; Duffner *et al.*, 2014). The adsorption of Zn on Fe oxides occurs by specific surface complexation (Liu *et al.*, 2016), which may be considered irreversible at pH > 6, and by non-specific adsorption, which may be also affected by pH (Montilla *et al.*, 2003). Surface complexes are also assumed to be the main mechanism of Zn adsorption on kaolinite (Wang *et al.*, 2017).

Humic substances (HS) behave as a natural "multiligand" complexing system (Buffle, 1988). The high selectivity of organic matter for most trace elements in their cationic forms indicates that the elements may form inner-sphere complexes with the carboxylic and hydroxyl functional groups (Senesi and Loffredo, 1998; Huang and Germida, 2002; Sparks, 2003; Li *et al.*, 2010). These inner-sphere complexes do not have interposed water molecules because ions are held together by bonds with a covalent character (Caporale and Violante, 2016). Studies carried out with inert substrates have demonstrated the ability of plants to assimilate micronutrients from metal-humic complexes (De Santiago and Delgado, 2007). The combined application of micronutrients and HS may reduce micronutrient deficiencies in soils (De Santiago *et al.*, 2008, 2011). Miravé and Orioli (1989) and Barnard *et al.* (1992) revealed that plants absorb Zn from Zn-HS complex. This explains the observed increases in plant growth after Zn-HS complex application in Zn-deficient soils (García-Mina *et al.*, 2004; Ozkutlu *et al.*, 2006). However, it is still not well understood how effective Zn-HS complex can be as a source of Zn to plants, as the process is governed by major geochemical reactive surfaces, *e.g.*, carbonates and Fe oxides.

Phytate (inositol-6-phosphate, IP_6) is the most abundant organic form of phosphorus (P) in soils (Turner *et al.*, 2002, 2003; Recena *et al.*, 2018) and soil organic amendments such as manure (Barberis *et al.*, 2003; He *et al.*, 2004a, b, 2006). The hydroxyl groups of IP_6 interact with metals such as Zn, leading to the formation of phytate salts (Marolt *et al.*, 2020). Despite its presence in soils, there is little information about the bioavailability of phytate-bound Zn.

In addition to the application of mineral Zn fertilizers to soil, there are other ways to mitigate Zn deficiency. Zinc chelates are highly efficient but expensive (Obrador *et al.*, 2003). In addition, their efficiency is reduced in calcareous soils due to their interaction with Fe and other soil components (López-Rayo *et al.*, 2015). Foliar spray improves the use efficiency of Zn fertilizers, but also increases application cost (Cakmak *et al.*, 2010a; Fu *et al.*, 2016). It was hypothesized that the application of Zn as HS or phytate complexes, which may be relatively stable in soil, would be more effective in overcoming Zn deficiency than the application of Zn as sulfate ($ZnSO_4$).

The main objective of this work was to study how Zn complexation by HS or phytate affects the uptake of Zn by wheat plants with the sorbent surfaces of carbonates and Fe oxides in growth medium. To this end, Zn-HS complex and Zn phytate were compared with the most common Zn fertilizer ($ZnSO_4$) in pot experiments using two types of growth media: siliceous sand coated with Fe oxide in the form of ferrihydrite (siliceous) and a minture of calcareous sand and siliceous sand (calcareous). This work will help to find effective strategies for Zn biofortification and control of Zn deficiency. In addition, the information obtained will contribute to a better understanding of the soil processes affecting Zn bioavailability. As carbonates (such as $CaCO_3$) and Fe oxides are ubiquitous soil components with strong affinity for Zn, it makes sense to study the effectiveness of different Zn sources in different growth media where Zn adsorption may be affected by Fe oxides or carbonates.

MATERIALS AND METHODS

Preparation of growth media and Zn sources

The siliceous sand and calcareous sand (> 99.5% $CaCO_3$) used were first sieved to select for particles between 0.2 and 0.5 mm to increase aeration and hydraulic conductivity of the growth media. The siliceous sand was washed with diluted Na_2CO_3 to disperse and remove clay minerals and Fe oxides, whereas the calcareous sand was washed with deionized water since it contained negligible Fe oxides and clay minerals. To prepare the siliceous growth medium, the siliceous sand was coated with Fe oxide following the procedure of Rahmatullah and Torrent (2000). Briefly, 50 mL 0.23 mol L^{-1} $Fe(NO_3)_3$ was added to 1 L 0.6 kg L^{-1} siliceous sand suspension. Then, pH was adjusted to 6 with 5 mol L^{-1} KOH to precipitate the ferrihydrite ($Fe_5O_8H \cdot nH_2O$) as shown in Fig. 1. The suspension was then heated to and maintained at 100 °C for 1 h to enhance the adsorption of Fe oxide on the sand particles. To prepare the calcareous growth medium, the siliceous sand was mixed with the calcareous sand at 3:1 (weight/weight). The main properties of the two growth media are detailed in Table I.

For preparation of Zn-HS complex, a commercial liquid mixture of humic and fulvic acids (Solfer húmicos®, Spain) was used. The mixture was dialyzed against deionized water using a 15-kDa cut-off Visking tubing (Sigma, Spain) until the electrical conductivity of the solution was < 1 dS m^{-1}. Then, the pH was adjusted to 8 with 1 mol L^{-1} HCl. The
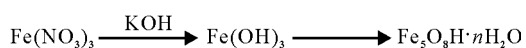
$$Fe(NO_3)_3 \xrightarrow{KOH} Fe(OH)_3 \longrightarrow Fe_5O_8H \cdot nH_2O$$

Fig. 1 Schematic showing the precipitation and transformation of ferrihydrite ($Fe_5O_8H \cdot nH_2O$) during the preparation of the Fe oxide-coated siliceous sand growth medium.

TABLE I

Properties[a] of the two growth media used in the two pot experiments

Growth medium	Fe_{CA}	ACCE	pH	EC
	$mg\ kg^{-1}$	%		$\mu S\ cm^{-1}$
A mixture of calcareous sand and siliceous sand	0	25	8.54	219
Fe oxide-coated siliceous sand	100	0	6.50	393

[a] Fe_{CA} = citrate ascorbate-extractable Fe; ACCE = active calcium carbonate equivalent; EC = electrical conductivity.

major properties of the dialyzed HS are shown in Table II. The Zn-HS complex was prepared using the dialyzed HS and 3 mmol L^{-1} $ZnSO_4$ solution at an HS:Zn stoichiometry of 2:0.65 and pH 9.2 (García-Mina *et al.*, 2004). The amount of HS to be combined with Zn was calculated based on the initial carbon concentration of HS. Zinc phytate was synthesized following Pierce (1985). Briefly, 0.05 mol L^{-1} IP_6 was mixed with 1 mol L^{-1} KCl and 1.5 mol L^{-1} $ZnSO_4$ following a 1:1:1 stoichiometry. The pH was adjusted to 5–6 with 1 mol L^{-1} KOH. The precipitate was filtered and oven-dried at 105 °C for 1 h.

TABLE II

Characteristics[a] of the dialyzed humic substances (molecular weight cut-off = 15 kDa) used for preparation of the Zn-humic substance complex

C	H	N	O	SUVA$_{254}$	E4/E6	P	Zn
	$g\ kg^{-1}$			$L\ g^{-1}\ cm^{-1}$		$mg\ kg^{-1}$	
360	88	$\leqslant 0.2$	552	80	3.24	nd[b]	nd

[a] SUVA$_{254}$ = specific ultraviolet absorbance at 254 nm; E4/E6 = ratio of measured spectral absorbance at 465 nm (E4) and to that at 665 nm (E6), determined according to Shirshova *et al.* (2006).
[b] Not detectable.

Experimental design

Two experiments using a completely randomized design with four replications were performed with the two growth media. The first experiment used the prepared Fe oxide-coated siliceous sand with Fe oxide in the form of ferrihydrite ($Fe_5O_8H \cdot nH_2O$) at a final concentration of 100 mg Fe kg^{-1}, measured as citrate ascorbate-extractable Fe (CA-Fe). The second experiment used the prepared calcareous growth medium.

In each experiment, three Zn sources were used: Zn-HS complex, Zn phytate, and $ZnSO_4$. For each Zn source, two Zn rates were set up: 0.25 and 2 mg kg^{-1} growth medium. Before applied in the Zn-HS treatments, the prepared Zn-HS was mixed with dialyzed Zn-free HS in such a way as to apply 0.1 g organic C kg^{-1} growth medium for each of the

two Zn rates. The high Zn rate (2 mg kg^{-1}) was chosen according to Liu *et al.* (2019), who estimated that the best Zn fertilization range is 5.7–11.4 kg ha^{-1}. The low Zn dose (0.25 mg kg^{-1}) was intended to test if Zn-HS complex and Zn phytate are more effective than $ZnSO_4$ even at low dose.

In each 350-mL cylindrical polystyrene pot (5.5-cm diameter, 15-cm height), 400 g growth medium was added. Seedlings (14–15-d old with three true leaves) of wheat (*Triticum durum* L. cv. Almicar) were transplanted to the pots at one seedling per pot and grown for 47 additional days till the end of the crop cycle. All Zn sources were applied with irrigation water in four irrigations (a total of 26 mL) during the first week of cultivation to avoid loss by drainage and to achieve a homogeneous distribution in the pot.

The experiments were conducted under controlled environmental conditions in a growth chamber, with a photoperiod of 14 h, a 25 °C/18 °C day/night temperature, 60% relative humidity, and a 22 W m^{-2} light intensity. These environmental conditions allowed us to finish the crop cycle in the time-frame stated above. The pots were watered daily with water or Zn-free Hoagland-type nutrient solution (one irrigation with water after four irrigations with nutrient solution). The nutrient solution had the following composition (concentration in mmol L^{-1}): $MgSO_4$ (2), KH_2PO_4 (1), $Ca(NO_3)_2$ (5), KNO_3 (5), KCl (0.05), Fe-EDDHA (0.02), H_3BO_3 (0.009), $MnCl_2$ (0.002 3), $CuSO_4$ (0.000 5), and H_2MoO_4 (0.000 5). Pots were irrigated at the soil surface to minimize drainage and the loss of nutrients. The total accumulated volume of nutrient solution applied per pot at the end of crop cycle was 240 mL, and that of water was 100 mL (including the 26 mL in the first week). During the experiment, potential adsorption of other nutrients on HS should not result in a differential effect between treatments since nutrients were continuously applied with nutrient solution. At the rates applied, HS and phytate should not affect the physical properties of the sandy growth media, either.

Plant analysis

At the end of the experiments, the whole plants were collected. Spikes, shoots, and roots of each plant were separated. The roots were washed first with deionized water then by ultrasound in deionized water for 1 min, rinsed again with deionized water, and dried on a filter paper. The plant samples were dried at 65 °C for 48 h in a forced-air oven. After weighing, the dry material was ground to pass a 1-mm screen, and an aliquot of 0.25 g was mineralized in a furnace at 550 °C for 8 h. The resulting ash was dissolved in 1 mol L^{-1} HCl, and the solution was heated at 100 °C for 15 min for full recovery of nutrients. The concentrations of Zn and P were determined by atomic absorption spectrometry (AAS) and the colorimetric method of Murphy and Riley (1962), respectively. Certified plant material (standard reference ma-

terial 1573a, National Institute of Standard and Technology, USA) was also analyzed to confirm full recovery of nutrients with this analytical procedure.

Total nutrient (*e.g.*, P and Zn) uptake by plants was calculated as the nutrient accumulation in aerial parts (spikes and shoots) and roots minus that in wheat seeds. Nutrient accumulation in each organ was calculated as the nutrient concentration multiplied by the dry biomass of the organ. The Zn harvest index (ZnHI) was calculated as the ratio of Zn in spikes to that in the aerial parts. At the end of the experiment, endogenous hormones (*i.e.*, auxins) in roots were determined with reverse-phase high performance liquid chromatography (HPLC) as described by Tang *et al.* (2011). For hormone extraction, fresh roots were crushed with liquid N_2 and subsequently homogenized. Three extractions (three consecutive days) were carried out with an 80% (volume/volume) methanol-water mixture in the dark overnight at $4\,^\circ$C. Extracts were centrifuged at $6\,000 \times g$ and $4\,^\circ$C for 15 min. The total extract per plant was evaporated and dissolved in 5 mL methanol. The phytohormones were analyzed by HPLC with a reverse-phase C18 column, and the mobile phase was methanol and 0.6% acetic acid at a volume ratio of 60:40 (Tang *et al.*, 2011).

Growth medium analysis

After harvesting, the rhizospheric growth media were separated from the roots by shaking. Following this, available Zn was determined using the diethylenetriaminepentaacetic acid (DTPA) method at pH 7.3 (Lindsay and Norvell, 1978). Sodium pyrophosphate extraction was performed to estimate the amount of Zn bound to organic matter (Loeppert and Inskeep, 1996). In these extracts, Zn was determined by AAS.

Electrical conductivity and pH were measured in water (saturation extract and 1:2.5 growth medium to water ratio, respectively). Olsen P in the growth media after harvesting was extracted with bicarbonate and determined colorimetrically (Olsen *et al.*, 1954; Murphy and Riley, 1962).

Statistical analysis

The effects of Zn source and rate on the different variables studied were assessed by two-way analysis of variance (ANOVA) using the software Statgraphics Plus 5.1 (StatPoint Technologies, Inc., USA). Differences between means were assessed by the least significant difference (LSD) test at $P < 0.05$. Normality and homoscedasticity were always ascertained by the Smirnoff-Kolmogorov and Levene tests, respectively (Acutis *et al.*, 2012), and data were power-transformed when one or both tests were not passed.

RESULTS

In the siliceous growth medium, the effect of each Zn

source on the dry biomass of spikes and shoots depended on the application rate, as revealed by the significant interaction between the two factors (Table III). The dry biomass yields of spikes and shoots with $ZnSO_4$ at the 2 mg kg^{-1} Zn rate were similar to those with Zn-HS complex at the 0.25 mg kg^{-1} Zn rate, and not significantly different from those obtained with Zn phytate at both rates and Zn-HS at the 2 mg kg^{-1} Zn rate. No significant effects of both factors on root were observed in this medium. Conversely, in the calcareous growth medium, Zn-HS complex and Zn phytate led to significantly higher dry biomass yields than $ZnSO_4$ (Table IV). The high Zn rate did not result in higher dry biomass yields in the calcareous medium. The concentration of auxins in roots showed lower values in the siliceous medium than in the calcareous medium, without significant differences between treatments in the siliceous medium (Table III). In the calcareous medium, auxin concentration in roots increased with increase in Zn rate when Zn was applied as Zn-HS complex or Zn phytate (Table IV).

In the siliceous growth medium, the interactive effect of the two factors on the Zn concentration in spikes was significant (Table III). Significantly higher Zn concentration in spikes was achieved with Zn-HS complex than that with Zn phytate or $ZnSO_4$ at the high Zn rate, while no significant differences between Zn sources were observed at the low Zn rate. In this medium, Zn-HS complex led to significantly higher Zn concentrations in shoots and roots than the other two Zn sources. In the calcareous growth medium, Zn-HS complex resulted in significantly higher Zn concentration in spikes and shoots than Zn phytate, while no significant difference was found in shoot Zn concentration between $ZnSO_4$ and Zn phytate (Table IV). The high Zn application rate always resulted in higher Zn concentrations in the three organs (spikes, shoots, and roots) in both media, except for roots in the calcareous medium (Tables III and IV).

In both growth media, Zn-HS complex led to significantly higher total Zn uptake than the other two sources, while no significant differences were observed between $ZnSO_4$ and Zn phytate (Fig. 2). In the siliceous growth medium, P uptake was not significantly different between treatments (Table III), but in the calcareous growth medium, Zn-HS complex and Zn phytate promoted significantly higher P uptake than $ZnSO_4$ (Tables III and IV).

The bioavailability of Zn in both growth media after harvest, measured as DTPA-extractable Zn (DTPA-Zn), was significantly higher with Zn-HS complex at the high rate than with the other treatments, while no significant differences were found between the three Zn sources at the low rate (Fig. 3). Zinc uptake by plants was linearly related with DTPA-Zn in the calcareous ($R^2 = 0.82$, $P < 0.001$) and siliceous growth media ($R^2 = 0.67$, $P < 0.001$) after harvest (data not shown). Organic matter-bound Zn,

TABLE III

Effects of Zn source and application rate on the dry biomass, Zn concentration, and Zn accumulation of spikes, shoots, and roots, auxin concentration in roots, P uptake, and Zn harvest index (ZnHI) of wheat in the pot experiment with Fe oxide-coated siliceous sand as the growth medium and summary of two-way analysis of variance (ANOVA) of the simple and interactive effects of the two factors

Zn source (S)	Zn rate (R)	Dry biomass			Root auxin concentration	ZnHI	Zn concentration			Zn accumulation			P uptake
		Spikes	Shoots	Roots			Spikes	Shoots	Roots	Spikes	Shoots	Roots	
	mg kg^{-1}	———— g plant^{-1} ————			µg kg^{-1}		———— mg kg^{-1} ————			———— µg plant^{-1} ————			mg plant^{-1}
Zn-HS[a)] complex	2	0.29bc[b)]	0.39ab	0.25	162	0.40	71.7a	81.4	105.1	20.5	32.2	25.2	2.13
	0.25	0.41ab	0.48a	0.24	166	0.53	20.0c	13.9	27.8	8.1	6.6	6.3	2.54
	Mean	0.35	0.43	0.24	164	0.46	45.8	47.6A[c)]	66.4A	14.3A	19.4A	15.7	2.33
Zn phytate	2	0.33abc	0.40ab	0.35	199	0.58	37.4b	22.0	24.2	12.1	9.2	7.8	2.33
	0.25	0.34abc	0.43ab	0.24	96	0.60	14.3c	8.2	23.6	4.9	3.5	6.5	2.36
	Mean	0.33	0.41	0.29	147	0.59	25.8	14.1B	23.9B	8.5B	6.3B	7.1	2.34
ZnSO$_4$	2	0.44a	0.48a	0.23	64	0.54	40.5b	35.5	45.8	18.2	16.8	10.2	2.22
	0.25	0.26c	0.27b	0.28	186	0.64	19.6c	9.3	16.8	5.5	2.4	4.6	2.12
	Mean	0.35	0.37	0.25	125	0.59	30.0	22.4B	31.3B	11.8AB	9.6B	7.4	2.17
ANOVA													
S		ns[d)]	ns	ns	ns	ns	***	*	*	*	***	ns	ns
R		ns	ns	ns	ns	ns	***	***	**	***	***	**	ns
S × R		*	*	ns	ns	ns	*	ns	ns	ns	ns	ns	ns

*, **, ***Significant at $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively.
[a)] Humic substance.
[b)] Means ($n = 4$) followed by different lowercase letters in a same column are significantly different according to the least significant difference (LSD) test at $P < 0.05$ when the interactive effect of Zn source × Zn rate is significant.
[c)] Different uppercase letters in a same column indicate that the means ($n = 8$) are significantly different between Zn sources according to the LSD test at $P < 0.05$ when the interactive effect of Zn source × Zn rate is not significant but the simple effect of Zn source is significant; regarding the significant effect of Zn rate, there is no indication of differences since there are only two levels of the factor.
[d)] Not significant.

TABLE IV

Effects of Zn source and application rate on the dry biomass, Zn concentration, and Zn accumulation of spikes, shoots, and roots, auxin concentration in roots, P uptake, and Zn harvest index (ZnHI) of wheat in the pot experiment with a mixture of calcareous sand and siliceous sand as the growth medium and summary of two-way analysis of variance (ANOVA) of the simple and interactive effects of the two factors

Zn source (S)	Zn rate (R)	Dry biomass			Root auxin concentration	ZnHI	Zn concentration			Zn accumulation			P uptake
		Spikes	Shoots	Roots			Spikes	Shoots	Roots	Spikes	Shoots	Roots	
	mg kg^{-1}	———— g plant^{-1} ————			µg kg^{-1}		———— mg kg^{-1} ————			———— µg plant^{-1} ————			mg plant^{-1}
Zn-HS[a)] complex	2	0.32	0.40	0.18	350a[b)]	0.48	54.6	52.3	31.3	17.6	21.9	4.6	1.66
	0.25	0.25	0.29	0.18	153cd	0.60	20.7	10.1	13.9	4.8	3.0	2.2	1.34
	Mean	0.28A[c)]	0.34A	0.18	251	0.54	37.6A	31.2A	22.6	11.2A	12.4A	3.4A	1.50A
Zn phytate	2	0.19	0.26	0.16	332ab	0.55	28.1	14.3	16.0	5.5	3.5	2.4	1.35
	0.25	0.29	0.38	0.15	78d	0.44	10.1	7.9	16.3	2.7	2.8	2.1	1.80
	Mean	0.24AB	0.32A	0.15	205	0.49	19.1B	11.1B	16.1	4.1B	3.1B	2.2AB	1.57A
ZnSO$_4$	2	0.12	0.21	0.15	190bc	0.48	37.2	16.0	15.0	5.5	4.8	2.5	0.60
	0.25	0.10	0.17	0.09	256abc	0.52	19.6	9.0	12.6	2.0	1.6	1.1	0.70
	Mean	0.11B	0.19B	0.12	223	0.5	28.4A	12.5AB	13.8	3.7B	3.2B	1.8B	0.65B
ANOVA													
S		*	*	ns[d)]	ns	ns	***	*	ns	***	*	*	**
R		ns	ns	ns	***	ns	***	***	ns	***	**	ns	ns
S × R		ns	ns	ns	**	ns	ns	ns	ns	ns	ns	ns	ns

*, **, ***Significant at $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively.
[a)] Humic substance.
[b)] Different uppercase letters in a same column indicate that the means ($n = 4$) are significantly different between Zn sources according to the least significant difference (LSD) test at $P < 0.05$ when the interactive effect of Zn source × Zn rate is not significant but the simple effect of Zn source is significant.
[c)] Different lowercase letters in a same column indicate that the means ($n = 8$) are significantly different according to the LSD test at $P < 0.05$ when the interactive effect of Zn source × Zn rate is significant; regarding the significant effect of Zn rate, there is no indication of differences since there are only two levels of the factor.
[d)] Not significant.

measured as pyrophosphate-extractable Zn, was significantly higher with Zn-HS complex at the high rate relative to the other treatments in the siliceous growth medium (Table V). For the calcareous medium, there was significant difference in pyrophosphate-extractable Zn between Zn-HS complex and ZnSO$_4$ but not between Zn-HS complex and Zn phytate. In this medium, the high Zn rate resulted in significant higher pyrophosphate-extractable Zn. Zinc source slightly affected
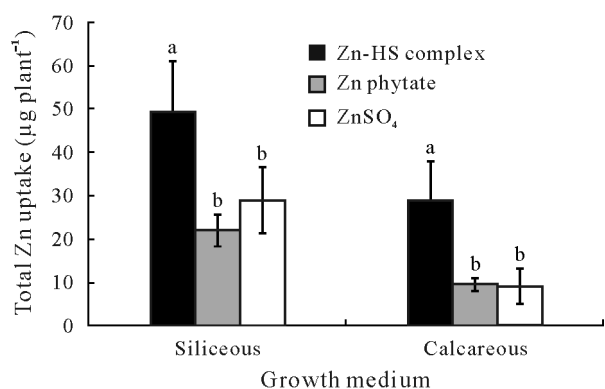
Fig. 2 Effects of the different Zn sources on total Zn uptake by wheat plants grown in the Fe oxide-coated siliceous sand (siliceous) growth medium and calcareous sand-siliceous sand mixture (calcareous) growth medium of pot experiments. Error bars are standard errors of the means ($n = 8$). Different letters indicate significant differences between Zn sources for a growth medium according to the least significant difference test at $P < 0.05$. HS = humic substance.

medium pH, with Zn-HS complex and Zn phytate leading to the highest pH values in the siliceous and calcareous medium, respectively.

DISCUSSION

Overall, Zn-HS complex led to the highest Zn concentration in spikes (biofortification effect), Zn uptake by plants, and Zn availability in the growth media. When Zn-HS complex was applied at the high rate, Zn concentration in the wheat grains (> 45 mg kg^{-1}) met the minimum Zn requirement for humans in both growth media (Pfeiffer and McClafferty, 2007; Cakmak, 2008; Wang *et al.*, 2020). In
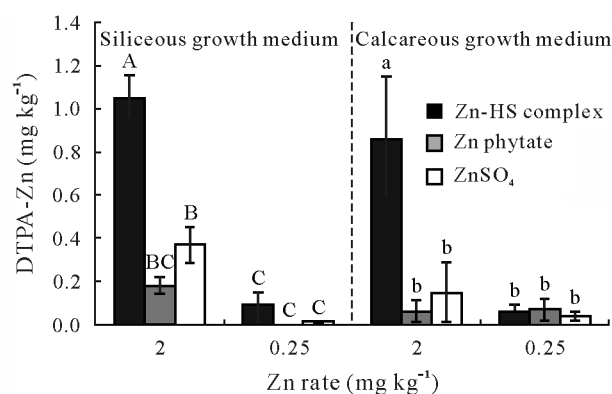

Fig. 3 Effects of Zn source and Zn application rate on the diethylene-triaminepentaacetic acid-extractable Zn (DTPA-Zn) content of the Fe oxide-coated siliceous sand (siliceous) growth medium and the calcareous sand-siliceous sand mixture (calcareous) growth medium of pot experiments. Each Zn source was applied at two Zn rates: 2 and 0.25 mg kg^{-1} medium. Error bars are standard errors ($n = 4$). Different uppercase letters indicate significant differences between treatments in the siliceous growth medium, whereas different lowercase letters indicate significant differences between treatments in the calcareous growth medium according to the least significant difference test at $P < 0.05$. HS = humic substance.

the siliceous medium, Zn-HS complex at the low rate and Zn phytate at both rates did not lead to significant differences in dry biomass yields of spikes and shoots when compared with the high rate of ZnSO$_4$ (Table III). In the calcareous medium, on average, Zn-HS complex and Zn phytate were significantly better than ZnSO$_4$ in promoting dry biomass yield (Table IV). All this suggests that application of Zn-HS complex or Zn phytate may effectively raise crop dry biomass in a Zn-deficient growth medium. Zinc rate did not affect the dry biomass of any plant part in the calcareous medium.

TABLE V

Effects of Zn source and application rate on medium pH, electrical conductivity (EC), Olsen P, and pyrophosphate-extractable Zn (Zn$_{pyro}$) in the pot experiments with Fe oxide-coated siliceous sand (siliceous) growth medium and calcareous sand-siliceous sand mixture (calcareous) growth medium and summary of two-way analysis of variance (ANOVA) of the simple and interactive effects of the two factors

Zn source (S)	Zn rate (R)	Siliceous medium				Calcareous medium			
		pH	EC	Olsen P	Zn$_{pyro}$	pH	EC	Olsen P	Zn$_{pyro}$
	mg kg^{-1}		μS cm^{-1}	—— mg kg^{-1} ——			μS cm^{-1}	—— mg kg^{-1} ——	
Zn-HS[a] complex	2	8.6	269	4.6	1.23	8.7	310	5.6	1.32
	0.25	8.6	254	5.3	0.17	8.8	318	3.4	0.33
	Mean	8.6A[b]	261B	4.9	0.7	8.7B	314	4.5	0.82A
Zn phytate	2	8.5	420	5.9	0	8.9	302	3.0	0.62
	0.25	8.4	487	2.9	0	8.8	298	3.4	0.22
	Mean	8.4AB	453A	4.4	0	8.8A	300	3.2	0.42AB
ZnSO$_4$	2	8.4	374A	2.9	0	8.8B	425	2.4	1.03B
	0.25	8.2	496	3.5	0	8.7	424	3.0	0.01
	Mean	8.3B	435A	3.2	0	8.7B	424	2.7	0.52B
ANOVA									
S		*	*	ns[c]	***	*	ns	ns	*
R		ns	ns	ns	***	ns	ns	ns	**
S × R		ns	ns	ns	***	ns	ns	ns	ns

*, **, ***Significant at $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively.
[a] Humic substance.
[b] Means ($n = 8$) followed by different uppercase letters in a same column are significantly different according to the least significant difference test at $P < 0.05$.
[c] Not significant.

However, best results in Zn concentration and uptake with all Zn sources were always obtained at the high Zn rate (2 mg kg^{-1}) in both media. This evidences that Zn needs to be applied at the high rate for a significant biofortification effect.

The effect of Zn-HS complex on plant growth was consistent with previous evidence suggesting its potential benefits on plant nutrition. We found that the effect of Zn-HS complex relative to the other Zn sources depended on the dominant Zn-adsorbing surface of the growth media. Only in the calcareous medium, Zn-HS complex performed better than ZnSO$_4$ in enhancing plant growth. This contradicted the result of Ozkutlu *et al*. (2006) who did not find differences between these two Zn sources in influencing wheat growth. Compared with the results of García-Mina *et al*. (2004) with a calcareous soil, the Zn-HS complex in this study displayed a positive effect on plant nutrition at much lower Zn rates. In addition, it is shown for the first time that Zn-HS complex had a greater biofortification effect than soluble mineral sources such as ZnSO$_4$.

In Zn-deficient plants, tryptophan synthesis is inhibited and consequently the production of auxins is decreased (Wood, 2007). In the calcareous medium, the increased auxins in roots with the high rates of Zn-HS complex and Zn phytate may be ascribed to the increased Zn concentration in plants. Increased auxins production explains the increased dry biomass yield and may enhance Zn translocation from roots to shoots (Olaetxea *et al*., 2018; Moreno-Lora *et al*., 2019). In addition, auxins accumulation in roots may reflect the need of an increased nutrient mobilization to overcome deficiencies since these hormones can activate the proton pumps (Tanimoto, 2005). This may explain the almost double concentration of auxins in the calcareous medium, where plants are more prone to micronutrient deficiency compared with the siliceous medium.

The highest Zn uptake with Zn-HS complex was because this source led to an increased Zn availability to plants in the growth media relative to the other Zn sources. This is evident in that, at harvest, near 50% of the Zn applied as Zn-HS complex remained extractable by DTPA, while less than 20% of the Zn applied as the other sources was recovered (Fig. 3). The high DTPA-Zn at harvest demonstrated that not only the uptake by plants but also the residual effect of Zn-HS complex was much higher than that of the other Zn sources.

Zinc salts (*e.g*., ZnSO$_4$) are soluble sources and their rapid release of Zn ions to the soil solution leads to the precipitation of Zn with carbonates (Rehman *et al*., 2018) and the subsequent adsorption on Fe oxides (Zhao *et al*., 2011). In contrast, complexation of Zn with HS may enable a slow and gradual release of Zn (Boguta and Sokołowska, 2016). The applied Zn-HS complex may remain, at least in part, complexed in the growth media. In fact, the stability constants of Zn-HS complex are high in the range of pH observed in this study (García-Mina *et al*., 2004). Such Zn-HS complex may constitute a source of available Zn to plants, which is not prone to precipitation or adsorption to the solid phase. This decreased precipitation and adsorption of Zn as Zn-HS complex explains the increased DTPA-Zn and Zn uptake by plants when compared with the other sources. In general, DTPA-Zn was lower in the calcareous medium than in the siliceous medium. However, the difference was much smaller with Zn-HS complex (*ca*. 20%) than with the other sources (*ca*. 63%). This indicates that Zn-HS complex had a greater effect on decreasing precipitation and adsorption of Zn on carbonates than on decreasing Zn adsorption on Fe oxides, which explains why Zn-HS complex performed better than ZnSO$_4$ in promoting Zn uptake and plant growth in the calcareous growth medium. The high pyrophosphate-extractable Zn with Zn-HS complex (Table V) supports that at least part of the Zn remained complexed by HS in the media. The basic pH of growth media may help to maintain Zn-HS complex in solution (Piccolo *et al*., 1993; Nardi *et al*., 1996), thus contributing to its use as Zn source by plants. As expected for ZnSO$_4$ and Zn phytate, their recovery with pyrophosphate was almost negligible in the siliceous medium. Surprisingly, there was a significant recovery by pyrophosphate with these Zn sources in the calcareous medium. This may be ascribed to the dispersion of Zn precipitates of colloidal size by pyrophosphate. According to Schindler (1991), the precipitation of Zn hydroxycarbonates, which may form colloidal particles, mainly rules the concentration of Zn in the aqueous phase of calcareous growth media.

An increased pH may contribute to a decreased Zn availability to plants. However, Zn-HS complex, which was the best source compared with the other sources, slightly increased the pH of the siliceous growth medium. Surface charge density of oxides may decrease with increasing pH as pH approaches the point of zero charge, and for Fe oxyhydroxides, this point is between pH 7.0 and 9.0 (Dzombak and Morel, 1990; Chesne and Kim, 2014). The decrease in surface charge may contribute to the decreased adsorption of Zn on Fe oxides and consequently the increased availability of Zn to plants.

In general, the plants absorbed about twice as much Zn in the siliceous medium compared with in the calcareous medium. This cannot be ascribed to pH difference since pH difference between media at the end of the experiment was minimal. It should be ascribed to the type of reaction with the dominant sorbent surface in each medium, *i.e*., carbonates or Fe oxides. Despite Zn adsorption on Fe oxides in the range of pH in this study can be considered irreversible, plant absorption and DTPA extraction of Zn were higher when these oxides were the dominant sorbent surfaces in comparison with the calcareous medium.

Zinc phytate showed a more stimulating effect on plant growth than ZnSO$_4$ in the calcareous medium (Table III). Phytate may form highly stable complex with Zn (Celi and Barberis, 2006) through coordination with its phosphate groups (Crea *et al.*, 2008). When Zn phytate is hydrolyzed by phosphohydrolase, the phosphate groups are removed and Zn is released to the aqueous solution (Lei and Porres, 2003). This progressive release of Zn to the solution may decrease its precipitation and adsorption rates. In addition, in P-poor calcareous media, the simultaneous release of inorganic P and Zn after phytate hydrolysis may decrease Zn adsorption (Wang and Harrel, 2005) and binding energy to sorbent surfaces (Saeed, 1977), thus increasing Zn availability to plants as observed by Recena *et al.* (2021). This may be reflected in the intermediate Zn concentration in roots with Zn phytate in the calcareous medium when compared with the other sources. The release of inorganic phosphate after hydrolysis may explain the increased P uptake with Zn phytate when compared with ZnSO$_4$ in the calcareous medium (Table IV). This effect was not observed in the siliceous medium likely because P availability was not so restricted by the precipitation of calcium phosphates as in the calcareous one. Similar results were observed with Zn-HS complex, likely due to a decreased rate of P precipitation by the effect of HS (Delgado *et al.*, 2002). Therefore, it can be concluded that Zn phytate, which is supposed to be present in soils, may be a source of Zn to plants. If applied as fertilizer, it may contribute to Zn and P supply to plants.

CONCLUSIONS

The complex Zn-HS performed significantly better than the other two Zn sources in supplying Zn to plants, independently of the main Zn sorbent surface (Fe oxides or carbonates) in the growth media. This is because Zn applied in the form of Zn-HS complex remained more available to plants than that applied as sulfate or phytate. Overall, plant dry biomass yield was significantly higher with Zn-HS complex and Zn phytate than with ZnSO$_4$ in the calcareous medium. Our findings suggest that Zn complexed to HS or phytate are not only sources of available Zn for plants, but also effective Zn fertilizers in particular when carbonates are the main Zn sorbent surface in the growth medium. The dominant Zn sorbent surface in growth media greatly influences the biofortification effect of Zn fertilizers.

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