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Zinc and phosphorus availability to wheat as affected by humic substances in calcareous and siliceous growth media

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Abstract

Aim of study: Humic substances (HS) have an impact on the dynamics of phosphorus (P) and zinc (Zn) in soil and consequently can affect the availability of both nutrients to plants. This work aimed to study the effect of humic substances on the availability of P and Zn to wheat depending on the main sorbent surfaces in growth media.

Area of study: Growth chambers of the Higher Technical School of Agricultural Engineering, University of Seville, Spain.

Material and methods: To this end, a pot experiment was performed involving three factors: i) HS rates, ii) Zn fertilization, and iii) type of growth medium, calcareous and noncalcareous (siliceous).

Main results: Biomass production and Zn uptake by plants decreased with increasing HS rates. Humic substances decreased Zn uptake more markedly in the siliceous medium. Negative effects of HS can be ascribed to altered crop nutrition and the high aromaticity of HS that can promote phytotoxic effects. The antagonistic effect between P and Zn was less evident in the calcareous medium than in the siliceous medium. This is probably explained by the reduced availability of Zn and the consequent decrease in uptake by plants in the calcareous medium compared to the siliceous medium. These differences observed between both media can be ascribed to different adsorption dynamics depending on the main sorbent surfaces.

Research highlights: The addition of HS, at the intermediate rates studied, had a positive effect on the microbial activity of the rhizosphere in the calcareous medium. Thus, not only crop functioning, but also soil biology, can be affected by the application of HS. This effect can be different depending on the HS rates applied and the type of growth medium.

Additional key words: iron oxides; carbonates; enzyme activity; micronutrients; bioavailability; oxalate; *Triticum aestivum*

Abbreviation used: DM (dry matter); DTPA (diethylene-triaminepentaacetic acid); EC (electrical conductivity); HS (humic substances); OM (organic matter); TPF (triphenyl formazan).

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Introduction

Zinc (Zn) is an essential micronutrient required by plants and animals (Alloway, 2009), whose deficiency in soils promotes yield losses in crops and reduces their quality for human consumption, especially in cereals (Rashid & Ryan, 2004; Ryan et al., 2013a). In Mediterranean regions, Zn deficiency is considered the most limiting nutritional disorder that affects crop production (Rashid & Ryan, 2004; Alloway, 2009). Since Zn has a strong affinity for calcium carbonate ($CaCO_3$) and iron (Fe) oxides, adsorption on these minerals is a crucial factor affecting its availability to plants. Basic pH promotes the formation of Zn-hydroxide forms (hydrozincite and Zn hydroxide), chemisorption on calcite ($ZnCO_3$) and co-precipitation in Fe oxides (Alloway, 2009; Ryan et al., 2013a; Rengel, 2015). Iron oxides can adsorb Zn through surface complexation processes, which are considered irreversible at basic pH. Adsorption can also occur through a nonspecific process depending on pH since the negative surface charges increase with increasing pH (Stahl & James, 1991; Buekers et al., 2007, 2008; Ryan et al., 2013a). Zinc adsorption increases with decreasing crystallinity of oxides and with increasing reaction time (Shuman, 1977; Buekers et al., 2008; Duffner, 2014). The main sorbing surfaces, carbonates or Fe oxides, in soils with basic pH need to be considered separately to obtain accurate predictions on the risk of Zn deficiency in plants (Imtiaz et al., 2006; Moreno-Lora & Delgado, 2020).

Phosphate can compete with Zn for adsorption sites (Madrid et al., 1991). On the other hand, Zn deficiency can be promoted in soils with elevated concentrations of P (Ryan et al., 2013b). There is an antagonistic effect between P and Zn that seems to vary depending on the minerals that absorb these nutrients (Sánchez-Rodríguez et al., 2013). In this regard, it has been suggested that P-induced Zn deficiency can increase with the ratio of Fe oxides to $CaCO_3$ (Rahmatullah & Torrent, 2000). Phosphorus can contribute to the decrease in grain quality since an increased concentration of phythate in grains, which is the main compound of P present in plant seeds, could bind to Zn inhibiting its absorption during digestion (Miller et al., 2007; Gharibzahedi & Jafari, 2017; Gómez-Coronado et al., 2019). Therefore, the equilibrium between these nutrients in soils and plants should be taken into account in order to overcome Zn deficiency and enrich cereal grains with Zn, the so-called biofortification. In this regard, the effects of soil management practices that affect P or Zn availability should be considered in an integrated way, as an increase in the availability of one nutrient can negatively affect the other.

Organic matter (OM) is an important factor affecting the availability of P and Zn to plants. Therefore, the management factors that affect organic matter in soil can change the availability of both nutrients to plants. As a dominant fraction of OM in the soil, the adsorption of humic substances (HS) on Fe oxides can alter that of P and Zn, affecting their concentration in solution and distribution in the soil (Rahmatullah & Torrent, 2000; De Santiago & Delgado, 2007). Humic substances can increase Zn adsorption on oxides (Güngör & Bekbölet, 2010; Stietiya & Wang, 2014). They can also reduce the precipitation of calcium phosphates (Delgado et al., 2002), increasing the concentration of P in the soil solution and therefore affecting that of Zn. In general, the interaction between P and Zn can be altered by HS because these substances have a metal complexing effect, can compete for adsorption sites, and can induce coadsorption processes. However, HS can promote negative effects on plants if applied at high rates due to phytotoxic effects (de Santiago et al., 2010). Thus, this work aimed at studying the effect of HS on P and Zn availability to plants and their interaction in two different growth media, i.e. siliceous and calcareous, with a view of assessing how the potential effect of HS vary depending on the main sorbent surfaces in the plant growth media. This will provide new insights on the role of OM in both the availability of nutrients and the efficiency of Zn fertilizer for overcoming its deficiency and for biofortification. Furthermore, the availability of Fe, Mn, and Cu will be assessed since these nutrients may interact with P and Zn and their availability can also be affected by HS.

Material and methods

Experimental design

A pot experiment was carried out in a growth chamber using wheat (*Triticum aestivum* L.) plants involving three factors: i) growth media, calcareous or non-calcareous (siliceous); ii) Zn fertilizer rate (1, 5, and 10 mg Zn kg^{-1} medium); and iii) HS rate (involving application of 0, 0.05, 0.1, and 0.5 g C kg^{-1} medium). Four replicates of each combination of treatments were used in the experiment in a complete randomized design.

Growth media

Two different growth media were prepared by mixing a basis of 2:3 (w:w) siliceous sand coated with Fe oxides (ferrihydrite) containing adsorbed P with i) 1:3 of siliceous sand (siliceous medium) or ii) 1:3 calcareous sand (calcareous medium). After homogenizing the mixture, 350 g of medium were placed in plastic pots (150 mm high and 55 mm diameter).

The siliceous sand was washed with distilled water and sodium carbonate, to disperse the colloids, and dried in a forced air oven at 48 °C. Siliceous sand coated with oxides was prepared following the procedure of Rahmatullah & Torrent (2000). The phosphorus was adsorbed to the ox-

Table 1. Properties of growth media

Medium	pH 1:5	EC (µS cm^{-1})	NO_3^- (mg L^{-1})	Olsen P (mg kg^{-1})	DTPA (mg kg^{-1})	
					Zn	Fe
Siliceous	5.58	63.55	14.33	12.15	0.03	6.67
Calcareous	8.60	111.17	20.67	17.08	0.03	4.71

EC: electrical conductivity. DTPA: diethylene-triaminepentaacetic acid

ides by immersing the coated sand in a solution containing 7 mg P L^{-1} (KH_2PO_4) at a ratio 1:1.5 (w:v) for 48 h. The composition of this solution, with the exception of P added, was the same as the solution used for irrigation and to supply nutrients (except for Zn, Fe, and P). The sand was carefully shaken every 6 h during this process. Then, the solution was removed, and the sand was washed with distilled water to remove P in the solution (not adsorbed), and the mixture dried in a forced air oven. Calcareous sand was washed and sieved to < 2 mm by decantation. To this end, 100 g of calcareous sand were placed in a 30 cm high cylinder filled with distilled water and settled for 38 s. Immediately after that, the suspended sand water was removed, and the settled sand was recovered and dried in a forced air oven. The siliceous and calcareous media were prepared by mixing the portions of each sand described above. Both media were analyzed in triplicate for their characterization (Table 1). Nitrate concentration was determined using test strips (Nitracheck, Merk). The nutritional availability indices of Fe and Zn (DTPA-extractable) (Lindsay & Norvell, 1978) and P (Olsen et al., 1954) were determined.

Plant material and growth conditions

Wheat seeds were disinfected by immersion in 5% NaClO for 1 min (de Santiago et al., 2011). Then, seeds were incubated in petri dishes at 6 ºC for a few days to induce germination. The plants were transferred to pots at the Z1.2 stage of the Zadoks scale (Zadoks et al., 1974).

The experiment was carried out in a growth chamber under controlled environmental conditions, with 16 h of light at 20 °C and 40% relative humidity and 8 h of darkness at 18 ºC and 60% relative humidity. Photosynthetic radiation (PAR) was 300 µmol photons $m^{-2} s^{-1}$. The plants were irrigated daily with a modified Hoagland nutrient solution without P, Fe, and Zn at pH 6.5, containing (mmol L^{-1}): $MgSO_4$ (2), $Ca(NO_3)_2$ (5), KNO_3 (5), KCl (0.05), H_3BO_3 (0.009), $MnCl_2$ (0.0023), $CuSO_4$ (0.0005) and H_2MoO_4 (0.0005). The supply of Zn was one of the factors in the experiment, and P and Fe were supposed to be supplied by the growth medium.

Treatments

Three doses of $ZnSO_4$ (low, medium and high) were dissolved in the nutrient solution and applied during the first three pot irrigations to apply a total dose of 1, 5, and 10 mg Zn kg^{-1} medium, respectively.

Four humic substances (HS) rates were applied based on carbon concentration (C) to achieve 0, 0.05, 0.1, and 0.5 g C kg^{-1} medium. Humic substances from a commercial product (Solfer Humicos, Lida Quimica) were dialysed prior to application to the growth medium. For dialysis, 50 mL of product was introduced into a dialysis membrane with a 14000-Da cutoff (Membra-cel, Serva), previously treated with 0.5 M NaOH, and placed in a container filled with distilled water. The water was renewed every 2 h during the first 12 h of the dialysis process and, after that, every 48 h until an electrical conductivity (EC) of the water lower than 100 µS cm^{-1}. Then, dialysed HS were collected. The density of the dialyzed HS was 1,003 kg L^{-1}, the pH 8.4, and the EC 944 µS cm^{-1}. The concentration of oxidable C was 0.358% (Walkley & Black, 1934) and total C was 0.3704% (Leco CNS analyzer, LECO Instrumentos, Madrid, Spain).

Sample collection and analyses

The pot experiment ended 77 days after transplanting, when plants reached stage Z9 (Zadoks et al., 1974). The plants were harvested and the shoots, roots, and grains separated. The roots were washed with distilled water and immersed in an ultrasonic bath for 1 min. Subsequently, all of this plant material was placed in a forced air oven at 65 °C for at least 48 h to determine the dry biomass.

Analysis of growth media

Rhizospheric medium was sampled as described by Wang et al. (2009), considering only that bound to the roots (Nazih et al., 2001). A portion of that was used for the determination of organic anions and enzyme activities and the other portion was air-dried and used to determine chemical parameters. All samples were stored at < 6 °C for less than 48 h before performing the organic anions and biochemical analysis. The concentration of organic anions was determined after extraction with 0.1 M NaOH (1:1) (Radersma & Grierson, 2004), following the chromatographic procedure of Gao et al. (2012). The β-glucosidase (Eivazi & Tabatabai, 1988) and alkaline phosphatase (Eivazi & Tabatabai, 1977) were determined colorimetrically by measuring the amount of p-nitrophenyl from the hydrolysis of p-nitrophenyl-β-glucopyranoside and p-nitrophenyl-phosphate, respectively. Dehydrogenase

Table 2. Analysis of variance (p values) of yield parameters and nutrient uptake in plants

Source of variation	Dry matter				Total uptake					ZnHI	PHI	P:Zn
	Total	Grain	Shoot	Root	P	Zn	Fe	Mn	Cu			
HS rate	0.000	0.000	0.016	0.001	0.000	0.000	0.000	0.000	0.000	0.727	0.804	0.000
Growth medium	0.000	0.000	0.000	0.000	0.000	0.000	0.079	0.000	0.000	0.686	0.003	0.053
Zn dose	0.061	0.542	0.177	0.013	0.106	0.000	0.006	0.011	0.143	0.119	0.418	0.000
HS*Medium	0.066	0.014	0.123	0.550	0.155	0.022	0.201	0.000	0.496	0.813	0.614	0.304
Zn*Medium	0.931	0.753	0.760	0.682	0.130	0.029	0.872	0.448	0.467	0.805	0.738	0.846
HS*Zn	0.773	0.156	0.329	0.413	0.096	0.266	0.361	0.041	0.385	0.798	0.304	0.650
HS*Zn*Medium	0.251	0.703	0.378	0.370	0.003	0.602	0.069	0.321	0.088	0.123	0.897	0.196

HS, humic substances. ZnHI and PHI: proportion of total nutrient in plants that is accumulated in grains. P:Zn, the P to Zn molar ratio in grain

was determined by measuring the concentration of triphenyl formazan (TPF) produced by enzymes (Casida et al., 1964). To this end, 2.5 g of medium, 0.02 g of $CaCO_3$, 0.8 mL of H_2O, and 0.3 mL of 3% 2,3,5-triphenyl-tetrazolium chloride (TTC) were placed in a falcon tube. This was mixed and incubated in the dark for 24 h. Subsequently, a volume of 3.9 mL of ethanol was added to extract the TPF produced. The mixture was centrifugated at 1258 *g* for 10 min. The amount of TPF in the supernatant was determined colorimetrically at 485 nm using a spectrophotometer. Nutrient availability indices of P (Olsen et al., 1954), Zn, and Fe (DTPA extraction according to Lindsay & Norvell (1978) were determined after cultivation to estimate the depletion of these nutrients during plant growth.

Plant analysis

The concentration and total amount of nutrients in each organ were determined. For this purpose, the plant material was ground to < 1 mm, and 0.25 g of each sample were calcinated in a muffle furnace, at 550 ºC for 8 h. To ensure the recovery of all elements, the ashes were dissolved in 10 mL of 1 M HCl at 100 °C for 15 min. The concentration of P in the resulting digest was determined colorimetrically according to Murphy & Riley (1962), while the micronutrients were measured by atomic absorption spectrometry. The total uptake of these nutrients by plants was calculated as the sum of the product of its concentration and the dry biomass in each organ. The nutrient harvest index was calculated as the ratio of nutrient accumulated in the grain to total uptake in the plant. The P:Zn molar ratio in grains was calculated as a quality trait of the grains.

Statistical analysis

A three-way analysis of variance was performed to assess the effect of the type of growth medium, Zn rate, and HS rate on the variables studied. Previously, normality according to the Shapiro-Wilks test, and homoscedasticity according to Levene's test, were verified. Power transformations were performed if necessary. The analysis of variance was carried out using the General Linear Model (GLM) procedure in Statgraphics 5.1 (StatPoint, 2000). When the effect of a factor was significant, the means for each factor level were compared according to Tukey's test ($p < 0.05$). When the interaction between factors was significant, the effect of the main factors couldn´t be evaluated, and the interaction was discussed, since the effect of one factor depends on the level of the other (Acutis et

Table 3. Analysis of variance (p values) of enzymatic activities and some parameters in the growth medium, after crop cycle.

Source of variation	Oxalic	Enzymatic activities			Olsen P	DTPA		pH	EC
		β-GLU	PHOS	DHA		Zn	Fe		
HS rate	0.000	0.109	0.883	0.000	0.000	0.000	0.000	0.000	0.000
Growth medium	0.000	0.559	0.000	0.001	0.000	0.002	0.000	0.000	0.000
Zn dose	0.658	0.018	0.559	0.956	0.001	0.000	0.197	0.000	0.638
HS*Medium	0.586	0.000	0.020	0.012	0.004	0.062	0.000	0.000	0.052
Zn*Medium	0.300	0.962	0.489	0.553	0.745	0.828	0.724	0.800	0.462
HS*Zn	0.616	0.065	0.300	0.887	0.442	0.004	0.332	0.000	0.02
HS*Zn*Medium	0.411	0.005	0.405	0.281	0.008	0.801	0.439	0.045	0.826

HS: humic substances. β-GLU: β-glucosidase activity. PHOS: alkaline phosphatase activity. DHA: dehydrogenase activity. DTPA: diethylene-triaminepentaacetic acid. EC: electrical conductivity.

Table 4. Effect of humic substances rate, growth medium type, and Zn dose on plants and oxalic concentration in the growth medium

Factor	Dry matter ($g\ plant^{-1}$)			Grain conc. ($mg\ kg^{-1}$)		Root conc. ($mg\ kg^{-1}$)			PHI	P:Zn	Total uptake		Oxalic ($mmol\ kg^{-1}$)
	Total	Grain	Shoot	Zn	Cu	P	Zn	Mn			Fe (mg)	Cu (µg)	
HS rate													
HS0	2.03a	0.93a	0.30a	29.3a	3.10a	518	59.9b	28.3c	0.85	104a	0.50a	8.33a	32.3a
HS1	1.96a	0.93a	0.28a	25.7b	2.50ab	479	58.3b	34.5bc	0.86	107a	0.37b	7.26ab	38.4a
HS2	1.86a	0.89ab	0.26a	25.6b	2.14b	482	58.8b	41.2b	0.84	108a	0.38b	6.97bc	38.3a
HS3	1.67b	0.83b	0.21b	30.3a	2.45ab	491	73.2a	55.0a	0.85	89b	0.21c	6.03c	23.6b
Zn dose													
Z1	1.89ab	0.89	0.25ab	23.0c	2.66	499	60.7	40.9	0.85	117a	0.33	7.22	32.2
Z2	1.80b	0.87	0.24b	27.5b	2.47	499	62.2	38.1	0.85	105b	0.32	6.75	33.3
Z3	1.95a	0.92	0.29a	32.4a	2.50	481	64.5	40.0	0.84	85b	0.44	7.46	33.8
Medium													
Calcareous	1.70b	0.78b	0.23b	25.4b	2.67	469b	59.2	24.5b	0.86a	105	0.33	6.40b	44.7a
Siliceous	2.08a	1.02a	0.30a	30.2a	2.41	518a	66.0	56a	0.84b	98	0.40	7.97a	20.7b

HS: humic substances. HS rates: HS0, HS1, HS2, and HS3 = 0, 0.05, 0.1, and 0.5 g C kg^{-1} medium, respectively. Zn rates: Z1, Z2, and Z3 = 1, 5, and 10 mg Zn kg^{-1} medium. PHI: proportion of total P in plant that is accumulated in grain. P:Zn, molar ratio of P to Zn in grain. Total uptake: the total uptake of nutrients by plant. Means followed by different letters in the same column for each level within a factor are significantly different according to the test of Tukey at $p < 0.05$.

al., 2012). In the case of double interaction, an ANOVA with the combined factors was performed and mean comparison was performed as described above. In the case of triple interaction, results were discussed on the basis of figures.

Results

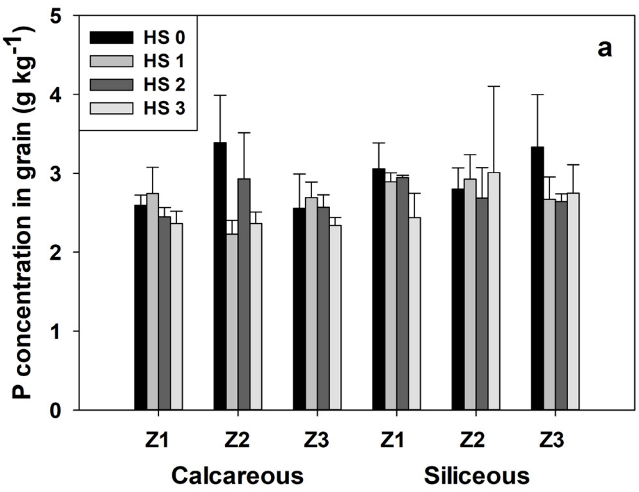
Effects on yield

In general, the application of HS affected most of the variables studied (Tables 2 and 3, and Table S1 [suppl]). However, in many cases, the effect of increasing HS rates varied depending on the other factors studied, as revealed by the significant interactions in the ANOVA. The growth medium and the dose of Zn also affected many of the variables studied. The siliceous medium outperformed the calcareous medium in terms of dry matter (DM) yield and nutrient uptake (some results shown). The DM in the plant organs, except for grains, decreased at increasing HS rates; however, only the highest rate promoted a significantly lower DM than the control without HS application (Table 4). In the case of grains, DM also decreased with increasing HS rates, but the decrease was more marked in the siliceous growth medium than in the calcareous growth medium, thus explaining the significant interaction between both factors (Tables 2 and 5).

The application of Zn at the highest dose favoured root development compared to the medium dose, but with no significant differences respect the lowest one (Table 4). Oxalate concentration in the rhizosphere was not affected by the Zn dose. The production of stem and grain biomass was not affected by this factor; therefore, the differences in the total biomass production between the Zn doses could be attributed to the development of the roots.

Effects on nutrient uptake

The total uptake of Fe and Cu clearly decreased at increasing HS rates (Table 4), meanwhile the uptake decreased with increasing HS rates more markedly in the siliceous growth medium (Table 5) than in the calcareous growth medium, thus explaining the interaction between both factors (Table 2). Manganese was the only nutrient studied whose uptake increased with increasing HS rates, although with different patterns in siliceous and calcareous growth media (Table 5). The concentration of Zn and Cu in grains decreased at intermediate HS rates, meanwhile Zn and Mn concentration in roots increased at the highest HS rate (Table 4). The concentration of Zn in the grain was much lower in the calcareous medium (Table 4). The Zn concentration in the grain increased with increasing Zn doses. However, there were no differences in the molar ratio P:Zn between the medium and the highest dose of Zn (Table 4). The concentration of Fe in grain and roots decreased with increasing HS rates in the calcareous medium. This was also observed for root Fe concentration in the siliceous medium; however, in this case, only intermediate HS rates decreased Fe concentration in grains relative to the control without HS (Table 5). Total P uptake was higher in the siliceous medium than in the calcareous one; in both media, it decreased with increasing doses of Zn (Fig. 1). However, this antagonistic effect was less marked in the calcareous growth medium. There was



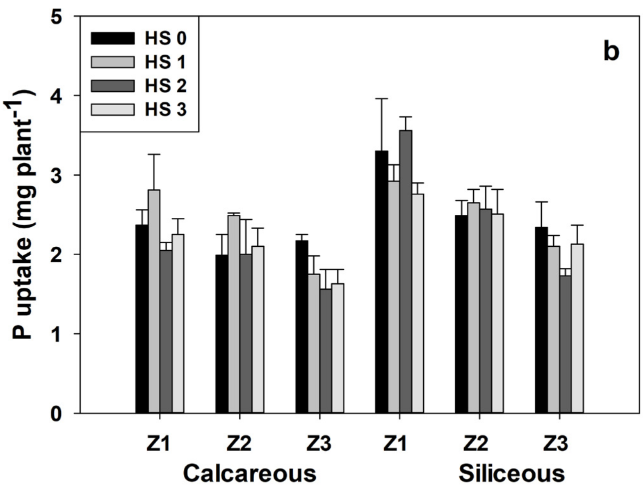


Figure 1. Effect of growth medium and Zn dose on P concentration in grain (a) and total P uptake by plants (b). HS rates: HS0, HS1, HS2, and HS3 = 0, 0.05, 0.1, and 0.5 g C kg^{-1} medium, respectively. Zn rates: Z1, Z2, and Z3 = 1, 5, and 10 mg Zn kg^{-1} medium, respectively. Error bars represent standar error (n=4).

no clear effect of increasing HS rates on total P uptake at the two lowest Zn doses; at the highest Zn dose, total P uptake tended to decrease with increasing HS rates (Fig. 1). The concentration of P in grains was less affected by Zn doses than total P uptake and tended to decrease with increasing HS doses, except in the siliceous medium at the intermediate Zn dose (Fig. 1). The P:Zn molar ratio in the grains decreased at the highest HS rate (Table 4).

Effect on the growth media

In both media, HS negatively affected oxalate concentration in the rhizosphere at the highest rate (Table 4) and the phosphatase activity in the calcareous medium (Table 5). In this medium, the dehydrogenase activity increased at intermediate HS rates, while it decreased at the highest HS rate in the siliceous medium (Table 5). Olsen P after crop decreased more in the calcareous than in the siliceous growth medium, and the effect of decreasing Olsen P by HS was less marked in the siliceous medium (Table S2 [suppl]). In both media, the DTPA extractable Fe after crop increased at increasing HS rates, although the trend was different between the calcareous and siliceous growth media (Table 5). However, the DTPA extractable Zn increased with increasing Zn doses, but only at the two highest doses. Intermediate HS rates promoted a decrease in DTPA extractable Zn (Fig. 2). Finally, the uptake of Zn by plants was related to DTPA extractable Zn in the growth medium; however, the relationship was different for both media (Fig. 3). The pH of the growth medium increased with increasing HS rate, although the trend was clearer in the calcareous growth medium (Table S2 [suppl]).

Table 5. Effect of the interaction between factors (humic substances rate and growth medium type) on studied variables

Medium	Enzyme activities		Fe-DTPA (mg kg-1)	DM grain (g plant^{-1})	Grain conc. (mg kg^{-1})		Root conc Fe (g kg^{-1})	Total uptake (µg)	
	PHOS (µg g^{-1} h^{-1})	DHA (ng g^{-1} h-^{1})			Fe	Mn		Zn	Mn
Calcareous									
HS0	34.1de	14.4b	3.62e	0.73bcd	7.77cd	10.61d	1.40abc	55.9bcde	24.0e
HS1	37.4cd	40.4a	3.19f	0.76b	7.85cd	12.14bc	1.43ab	52.3cde	30.5de
HS2	34.3de	47.1a	3.05f	0.68cd	5.98cd	12.85b	1.26bcd	49.7de	29.5de
HS3	30.4e	14.9b	4.76d	0.61e	5.46d	13.03b	0.87ef	47.4e	42.7bc
Siliceous									
HS0	40.9abc	22.8b	6.08c	0.90a	13.00a	15.85a	1.52a	82.5a	57.4ab
HS1	38.9bcd	23b	6.95b	0.79b	8.46bc	10.38d	1.08de	63.1b	37.3cd
HS2	43.6ab	19b	7.21b	0.75bc	6.39cd	10.82cd	1.21cd	60.7bc	50.8ab
HS3	44.7a	7.6c	9.93a	0.65de	12.22ab	16.14a	0.69f	59.3bcd	72.7a

HS: humic substances. HS rates: HS0, HS1, HS2, and HS3 = 0, 0.05, 0.1, and 0.5 g C kg medium^{-1}, respectively. PHOS: alkaline phosphatase activity. DHA: dehydrogenase activity. DTPA: diethylene-triaminepentaacetic acid. DM: dry matter. Total uptake: the total uptake of nutrients by plant. Means followed by different letters in the same column are significantly different according to the test of Tukey at $p < 0.05$.

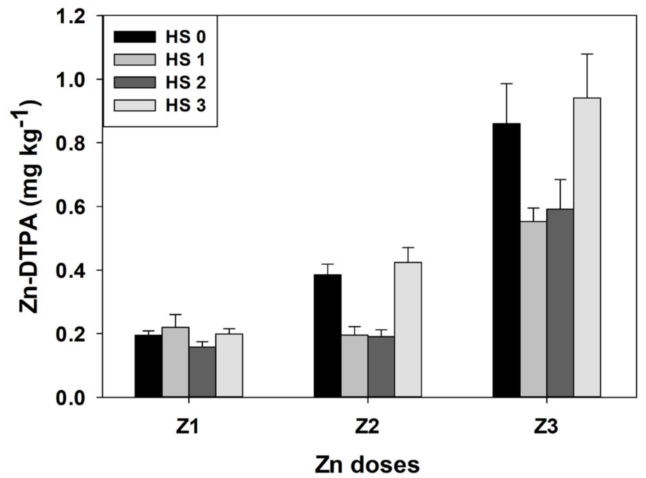


Figure 2. Effect of Zn doses and humic substances rate (HS) on Zn availability after crop cycle (Zn-DTPA). HS rates: HS0, HS1, HS2, and HS3 = 0, 0.05, 0.1, and 0.5 g C kg^{-1} medium, respectively. Zn rates: Z1, Z2, and Z3 = 1, 5, and 10 mg Zn kg^{-1} medium, respectively. Error bars represent standar error (n=8).

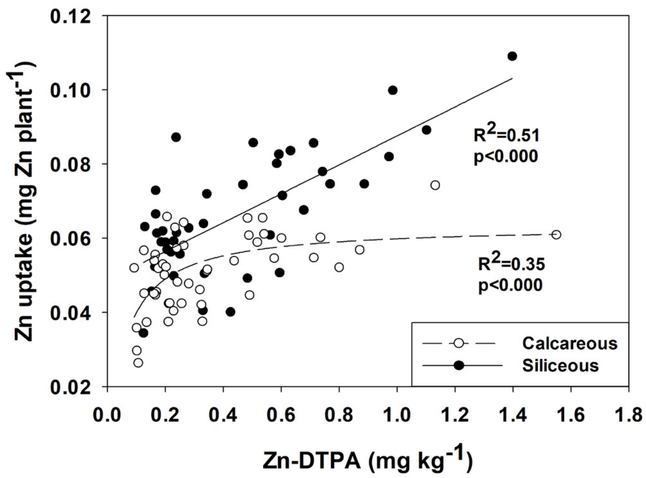


Figure 3. Relationship between Zn uptake and Zn availability after crop cycle (Zn-DTPA) in calcareous and siliceous growth medium. For the whole data set, the relationship was also significant (Y=0.046+0.031X; R^2=0.36; p<0.000).

Discussion

In general, biomass production and nutrient uptake, except for Mn, decreased with increasing HS rates, regardless of growth medium. Thus, it cannot be ascribed to particular reactions of applied HS or nutrients in each growth medium. These results agree with de Santiago et al. (2010) who observed adverse effects on lupines at HS concentrations higher than 0.1 g C kg^{-1} soil, ascribed at least in part to the high aromaticity of HS that promoted phytotoxic effects. The root was the organ where the highest inhibition of development was observed (by 30%) with the highest rate of HS. This likely revealed this phytotoxic effect. This reduction in root development explains the lower concentration of oxalate in the rhizosphere, since this production is partly due to root exudations that will decrease with less root development. Exudation of organic anions is a mechanism for nutrient mobilization in plants and microorganisms, especially for metals and P (García-López & Delgado, 2016). The increase in Zn and Mn concentration in roots with increasing HS rates could be explained by a concentration effect given the lower root development at this rate. In contrast, the concentration of Fe in the roots decreased markedly with increasing HS rates. In addition to decreased oxalate exudation, potential adsorption of HS on Fe oxides and/or complexation of Fe by HS can negatively affect Fe mobilization from the growth medium and absorption by plants, thus explaining this negative effect on Fe nutrition.

Without HS application, Zn uptake by plants was higher in siliceous than in calcareous growth medium. This reveals that carbonates may constrain Zn availability more than Fe oxides, or Zn adsorption on Fe oxides may be less reversible in the presence of carbonates. In calcareous soils, Zn precipitated or adsorbed on carbonates is not available to plants (Imtiaz et al*.,* 2006). However, with increasing HS rates, there was no significant effect on decreasing Zn uptake by plants in calcareous growth medium while this decrease was significant in the case of siliceous growth medium (Table 5). This reveals more negative effect of HS when the unique Zn sorbent surface is Fe oxides, perhaps due to an increased adsorption of Zn on Fe oxides promoted by HS (Stietiya & Wang, 2014). Zinc complexed by HS can be a source of nutrients for plants (García-López et al*.,* 2022). Thus, it seems that the effect of HS decreasing Zn uptake may be ascribed to the interaction of the nutrient with the sorbent surfaces, decreasing its activity in solution.

The Zn availability index, i.e. Zn extractable by DTPA, increased with the Zn dose applied to the media. The addition of humic substances apparently impaired this index compared to the control at the two lowest Zn doses; however, there was no difference between the control and the highest HS rate (Fig. 2). This is probably due to the interaction with sorbent surfaces and the formation of complexes between humic substances and Zn. At low HS rates, HS can induce a higher Zn adsorption on oxides and carbonates, while at the highest HS rate, part can remain in solution as Zn complexes that can be a source of available Zn to plants (García-López et al., 2022). However, the ratio between DTPA extractable Zn and Zn absorbed by the plant was different depending on the growth medium, with higher ratios found for the siliceous growth medium (Fig. 3). Therefore, the accuracy of the Zn availability index should take into account the main reactions involved in Zn retention in soils.

Humic substances had positive effects on Mn uptake by plants, in agreement with Çelik et al. (2010). This could be associated with its complexation with humic substances. It should be noted that the negative effect of increasing HS rates on the concentration of Fe and its total uptake disagreed with the effect on DTPA extractable Fe, which showed the opposite trend. This is not surprising since DTPAextractable Fe cannot be considered an accurate index of Fe availability, particularly in calcareous soils (de Santiago & Delgado, 2006). Thus, HS increases the chemical extraction through complexation of Fe, but not its real availability to plants. Probably, the adsorption of HS on Fe oxides may decrease the efficiency of phytosiderophores produced by wheat roots by competing for adsorption sites on oxides, decreasing the mobilization of the nutrient from oxides.

The effect of HS on P nutrition varied depending on the growth medium and the dose of Zn (Table 2 and Table S1 [suppl]). There was a clear antagonistic effect of Zn on P uptake that was less marked in the calcareous growth medium (Fig. 1). This contradicts the results of Chen et al. (2018), who showed P-Zn antagonism with a P concentration 20 times higher in acid soil than in calcareous soil. In our case, the decreased availability of Zn in the calcareous medium led not only to a decreased Zn uptake by plants but also to a decreased antagonistic effect on P. In addition, the calcareous medium had an initial Olsen P higher than that of the siliceous medium. Thus, with higher availability of P, the potential antagonism induced by Zn could be less marked. There is no negative effect of HS on P uptake except for the highest Zn rate in both media. Humic substances are known to increase P availability (Delgado et al., 2002). However, when the antagonism with Zn is more marked, it seems that HS contributes negatively to the availability of P to plants.

The plants grown in the siliceous medium showed a higher concentration of Zn in the grain than those grown in the calcareous medium. This helps to explain that the P:Zn molar ratio tended to be lower in the siliceous medium than in the calcareous one ($p < 0.053$). Thus, the growth medium has a clear effect on the quality of the grain in terms of the Zn concentration and digestibility. On the other hand, HS reduced grain yield, increasing the concentration of Zn in the grain by concentration effect at the highest HS rate. Consequently, despite the decrease in yield, the P:Zn molar ratio of grains was decreased.

To cope with low Zn availability, especially in alkaline soils, the plant excretes organic acids, which lower soil pH, thus increasing Zn availability (Bouain et al., 2014). However, no significant differences in oxalate production were found between the Zn doses to support this hypothesis. Experiments carried out by Duffner et al. (2012) also did not show increased root exudation of organic anions in response to low Zn availability.

The addition of the two intermediate rates of HS increased the activity of dehydrogenase in the calcareous medium by more than 65% compared to the control without HS; the highest rate of HS did not show significant differences with the control (Table 5). Since this enzyme activity has a microbial origin and is intracellular, it can be assumed that HS were a source of C for microorganisms. In contrast, in the siliceous medium, the intermediate levels did not improve the dehydrogenase activity, and a negative effect was observed at the highest rate compared to the control. In this medium, the stabilization of HS through adsorption of Fe oxides should make them less accessible for microbial degradation, thus explaining the lack of a positive effect of HS on microbial enzymes. In the calcareous medium, the increase in pH due to the effect of carbonates can lead to a decrease in HS adsorption (Avena & Koopal, 1998) making HS more accessible to microorganisms. At the highest rate, HS may have negative effects on rhizospheric microorganisms. It is known that the microbial activity in the rhizosphere and consequently the dehydrogenase activity increase with increasing plant root development (Woli et al., 2012). Therefore, the phytotoxic effect on plants can lead to a decrease in microbial activity in the rhizosphere. Phosphatase activity showed a different pattern from that of dehydrogenase, with no clear negative effect of HS. It should be noted that this activity is also associated with the plant and not only with microorganisms such as dehydrogenase.

In summary, increasing HS rates negatively affected Zn nutrition in siliceous medium without significant effects in calcareous medium, moreover it had little effect on P, and improved Mn nutrition. The different effects on the availability of Zn to plants, depending on the growth medium, can be ascribed to different adsorption dynamics, depending on the main sorbent surfaces. Impaired Zn and Fe nutrition contributes to explain, in addition to a phytotoxic effect, the negative effect on plant DM yield at the highest HS rate. The grain yield decreased with increasing HS rates more markedly in the siliceous medium than in the calcareous medium. However, the addition of HS, at the intermediate rates studied, had a positive effect on the microbial activity of the rhizosphere in calcareous media. Thus, not only crop functioning, but also soil biology, can be affected with the application of HS. This effect can be different depending on the HS rates applied and the type of growth medium.

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References

Acutis M, Scaglia B, Confalonieri R, 2012. Perfunctory analysis of variance in agronomy, and its consequences in experimental results interpretation. Eur J Agron 43: 129-135. https://doi.org/10.1016/j.eja.2012.06.006

Alloway BJ, 2009. Soil factors associated with zinc deficiency in crops and humans. Environ Geochem Health 31(5): 537-548. https://doi.org/10.1007/s10653-009-9255-4

Avena MJ, Koopal LK, 1998. Desorption of humic acids from an iron oxide surface. Environ Sci Technol 32(17): 2572-2577. https://doi.org/10.1021/es980112e

Bouain N, Shahzad Z, Rouached A, Khan GA, Berthomieu P, Abdelly C, et al., 2014. Phosphate and zinc transport and signalling in plants: Toward a better understanding of their homeostasis interaction. J Exp Bot 65(20): 5725-5741. https://doi.org/10.1093/jxb/eru314

Buekers J, Van Laer L, Amery F, Van Buggenhout S, Maes A, Smolders E, 2007. Role of soil constituents in fixation of soluble Zn, Cu, Ni and Cd added to soils. Eur J Soil Sci 58(6): 1514-1524. https://doi.org/10.1111/j.1365-2389.2007.00958.x

Buekers J, Amery F, Maes A, Smolders E, 2008. Long-term reactions of Ni, Zn and Cd with iron oxyhydroxides depend on crystallinity and structure and on metal concentrations. Eur J Soil Sci 59(4): 706-715. https://doi.org/10.1111/j.1365-2389.2008.01028.x

Casida LE, Klein DA, Santoro T, 1964. Soil dehydrogenase activity. Soil Sci 98(6): 371-376. https://doi.org/10.1097/00010694-196412000-00004

Çelik H, Katkat AV, Aşık BB, Turan MA, 2010. Effect of foliar-applied humic acid to dry weight and mineral nutrient uptake of maize under calcareous soil conditions. Commun Soil Sci Plant Anal 42(1): 29-38. https://doi.org/10.1080/00103624.2011.528490

Chen XX, Zhang W, Wang Q, Liu YM, Liu DY, Zou CQ, 2018. Zinc nutrition of wheat in response to application of phosphorus to a calcareous soil and an acid soil. Plant Soil 434(1-2): 139-150. https://doi.org/10.1007/s11104-018-3820-5

de Santiago A, Delgado A, 2006. Predicting iron chlorosis of lupin in calcareous Spanish soils from iron extracts. Soil Sci Soc Am J 70(6): 1945. https://doi.org/10.2136/sssaj2005.0343

de Santiago A, Delgado A, 2007. Effects of humic substances on iron nutrition of lupin. Biol Fertil Soils 43(6): 829-836. https://doi.org/10.1007/s00374-007-0191-0

de Santiago A, Expósito A, Quintero JM, Carmona E, Delgado A, 2010. Adverse effects of humic substances from different origin on lupin as related to iron sources. J Plant Nutr 33(2): 143-156. https://doi.org/10.1080/01904160903434220

de Santiago A, Quintero JM, Avilés M, Delgado A, 2011. Effect of Trichoderma asperellum strain T34 on iron, copper, manganese, and zinc uptake by wheat grown on a calcareous medium. Plant Soil 342(1-2): 97-104. https://doi.org/10.1007/s11104-010-0670-1

Delgado A, Uceda I, Andreu L, Kassem S, Del Campillo MC, 2002. Fertilizer phosphorus recovery from gypsum-amended, reclaimed calcareous marsh soils. Arid L Res Manag 16(4): 319-334. https://doi.org/10.1080/15324980290000421

Duffner A, Hoffland E, Temminghoff EJM, 2012. Bioavailability of zinc and phosphorus in calcareous soils as affected by citrate exudation. Plant Soil 361(1-2): 165-175. https://doi.org/10.1007/s11104-012-1273-9

Duffner A, 2014. Chemical and biological rhizosphere interactions in low zinc soils. Doctoral thesis. Wageningen University, The Netherlands.

Eivazi F, Tabatabai MA, 1977. Phosphatases in soils. Soil Biol Biochem 9(3): 167-172. https://doi.org/10.1016/0038-0717(77)90070-0

Eivazi F, Tabatabai MA, 1988. Glucosidases and galactosidases in soils. Soil Biol Biochem 20(5): 601-606. https://doi.org/10.1016/0038-0717(88)90141-1

Gao Z, Shao J, Sun H, Zhong W, Zhuang W, Zhang Z, 2012. Evaluation of different kinds of organic acids and their antibacterial activity in Japanese apricot fruits. Afr J Agric Res 7(35): 4911-4918. https://doi.org/10.5897/AJAR12.1347

García-López AM, Delgado A, 2016. Effect of Bacillus subtilis on phosphorus uptake by cucumber as affected by iron oxides and the solubility of the phosphorus source. Agric Food Sci 25(3): 216-224. https://doi.org/10.23986/afsci.56862

García-López AM, Recena R, Delgado A, 2022. Effect of Zn binding to phytate and humic substances on its uptake by wheat (*Triticum durum* L.) as affected by carbonates and Fe oxides. Pedosphere 32(6): 823-832. https://doi.org/10.1016/j.pedsph.2022.06.022

Gharibzahedi SMT, Jafari SM, 2017. The importance of minerals in human nutrition: Bioavailability, food fortification, processing effects and nanoencapsulation. Trends Food Sci Technol 62: 119-132. https://doi.org/10.1016/j.tifs.2017.02.017

Gómez-Coronado F, Almeida AS, Santamaría O, Cakmak I, Poblaciones MJ, 2019. Potential of advanced breeding lines of bread-making wheat to accumulate grain minerals (Ca, Fe, Mg and Zn) and low phytates under Mediterranean conditions. J Agron Crop Sci 205(3): 341-352. https://doi.org/10.1111/jac.12325

Güngör EBO, Bekbölet M, 2010. Zinc release by humic and fulvic acid as influenced by pH, complexation and DOC sorption. Geoderma 159(1-2): 131-138. https://doi.org/10.1016/j.geoderma.2010.07.004

Imtiaz M, Alloway B, Aslam M, Memon M, Khan P, Siddiqui SUH, et al., 2006. Zinc sorption in selected soils. Commun Soil Sci Plant Anal 37(11-12): 1675-1688. https://doi.org/10.1080/00103620600710330

Lindsay WL, Norvell WA, 1978. Development of a DTPA soil test for zinc, iron, manganese, and copper. Soil Sci Soc Am J 42: 421-428. https://doi.org/10.2136/sssaj1978.03615995004200030009x

Madrid L, Diaz-Earrientos E, Contreras MC, 1991. Relationships between zinc and phosphate adsorption on montmorillonite and an iron oxyhydroxide. Aust J Soil Res 29(2): 239-247. https://doi.org/10.1071/SR9910239

Miller LV, Krebs NF, Hambidge KM, 2007. A mathematical model of zinc absorption in humans as a function of dietary zinc and phytate. J Nutr 137(1): 135-141. https://doi.org/10.1093/jn/137.1.135

Moreno-Lora A, Delgado A, 2020. Factors determining Zn availability and uptake by plants in soils developed under Mediterranean climate. Geoderma 376: 114509. https://doi.org/10.1016/j.geoderma.2020.114509

Murphy J, Riley JP, 1962. A modified single solution method for the determination of phosphate in natural waters. Anal Chim Acta 27: 31-36. https://doi.org/10.1016/S0003-2670(00)88444-5

Nazih N, Finlay-Moore O, Hartel PG, Fuhrmann JJ, 2001. Whole soil fatty acid methyl ester (FAME) profiles of early soybean rhizosphere as affected by temperature and matric water potential. Soil Biol Biochem 33(4-5): 693-696. https://doi.org/10.1016/S0038-0717(00)00197-8

Olsen SR, Cole CV, Watanabe FS, Dean LA, 1954. Estimation of available phosphorus in soils by extraction with sodium bicarbonate. USDA 939, 19 pp. https://doi.org/10.2307/302397

Radersma S, Grierson PF, 2004. Phosphorus mobilization in agroforestry: Organic anions, phosphatase activity and phosphorus fractions in the rhizosphere. Plant Soil 259(1-2): 209-219. https://doi.org/10.1023/B:PLSO.0000020970.40167.40

Rahmatullah, Torrent J, 2000. Phosphorus dynamics and uptake by wheat in a model calcite-ferrihydrite system. Soil Sci 165(10): 803-812. https://doi.org/10.1097/00010694-200010000-00005

Rashid A, Ryan J, 2004. Micronutrient constraints to crop production in soils with mediterranean-type characteristics: A review. J Plant Nutr 27(6): 959-975. https://doi.org/10.1081/PLN-120037530

Rengel Z, 2015. Availability of Mn, Zn and Fe in the rhizosphere. J Soil Sci Plant Nutr 15(2): 397-409. https://doi.org/10.4067/S0718-95162015005000036

Ryan J, Rashid A, Torrent J, Yau SK, Ibrikci H, Sommer R, et al., 2013a. Micronutrient constraints to crop production in the middle east-west Asia region: Significance, research, and management. Adv Agron 122: 1-84. https://doi.org/10.1016/B978-0-12-417187-9.00001-2

Ryan J, Ibrikci H, Rashid A, Sommer R, 2013b. Phosphorus in low-input dryland agriculture: The perspective from Syria. Commun Soil Sci Plant Anal 44(16): 2378-2392. https://doi.org/10.1080/00103624.2013.794825

Sánchez-Rodríguez AR, Cañasveras JC, del Campillo MC, Barrón V, Torrent J, 2013. Iron chlorosis in field grown olive as affected by phosphorus fertilization. Eur J Agron 51: 101-107. https://doi.org/10.1016/j.eja.2013.07.004

Shuman LM, 1977. Adsorption of Zn by Fe and Al hydrous oxides as influenced by aging and pH1. Soil Sci Soc Am J 41(4): 703. https://doi.org/10.2136/sssaj1977.03615995004100040016x

Stahl RS, James BR, 1991. Zinc sorption by B horizon soils as a function of pH. Soil Sci Soc Am J 55: 1592-1957. https://doi.org/10.2136/sssaj1991.03615995005500060015x

Stietiya MH, Wang JJ, 2014. Zinc and cadmium adsorption to aluminum oxide nanoparticles affected by naturally occurring ligands. J Environ Qual 43(2): 498-506. https://doi.org/10.2134/jeq2013.07.0263

Walkley A, Black IA, 1934. An examination of the degtjareff method for determining soil organic matter, and a proposed modification of the chromic acid titration method. Soil Sci 37(1): 29-38. https://doi.org/10.1097/00010694-193401000-00003

Wang G, Xu Y, Jin J, Liu J, Zhang Q, Liu X, 2009. Effect of soil type and soybean genotype on fungal community in soybean rhizosphere during reproductive growth stages. Plant Soil 317(1-2): 135-144. https://doi.org/10.1007/s11104-008-9794-y

Woli A, St Z, Wolińska A, Stępniewska Z, 2012. Dehydrogenase activity in the soil environment. Dehydrogenases: 183-210. https://doi.org/10.5772/48294

Zadoks JC, Chang TT, Konzak CF, 1974. A decimal code for the growth stages of cereals. Weed Res 14(6): 415-421. https://doi.org/10.1111/j.1365-3180.1974.tb01084.x