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REGULAR ARTICLE

Zinc biofortification strategies for wheat grown on calcareous Vertisols in southern Spain: application method and rate

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Abstract
Purpose The aims of this work were (i) to find a soil indicator to predict durum wheat yield response to Zn fertilization, (ii) to compare the effect of various Zn fertilization strategies on wheat yield and Zn biofortification in calcareous Vertisols of southern Spain, and (iii) to assess the effect of these Zn fertilization strategies on crop P uptake (durum and bread wheat).
Methods Different Zn fertilization strategies, soil application (0.3–10 kg ha^{-1}) and foliar spraying (two rates, different growth stages), were tested in wheat crops under field conditions in the period 2012–2019.
Results A simple soil indicator failed to predict durum wheat response to Zn fertilization. Only one of the combinations tested increased wheat yield in the 11 field experiments carried out. Zinc foliar spraying (1.28 kg ha^{-1}) was effective for wheat biofortification when applied at early booting (durum wheat) or flowering, and also when splitting this application between stem elongation and flowering stages (bread wheat). The foliar treatments produced the highest zinc use efficiencies (6–19%) and soil applications the lowest (0.2–1.3%). Moreover, foliar treatments increased grain Zn concentrations by 12–51% while soil application increased such concentrations by only 4–13%. None of the Zn fertilization strategies altered P uptake.
Conclusion No yield increase in wheat is expected from Zn fertilization for the application methods and rates used here and the soils studied (calcareous Vertisols under Mediterranean climate). However, foliar applications at and after early booting stage are promising for durum and bread wheat biofortification.

Keywords P and Zn interaction · Zn uptake · Foliar spraying · Soil application · Biofortification · Zinc use efficiency

Abbreviations
EC electrical conductivity

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OC	organic carbon by rapid dichromate oxidation
P_{Olsen}	Available soil P
Zn_{DTPA}	labile Zn in soil, extracted with diethylenetriaminepentaacetic acid
ZnUE	Zn use efficiency

Introduction

Micronutrient deficiencies have recently become a relevant health issue for humans, especially in developing countries, as they now affect more than 30% of the world's population (Welch et al. 2013). A good example of this is zinc (Zn) deficiency, which has been estimated to be present in more than one-half of the global population by effect of a low Zn dietary intake (Nriagu 2019). A reduced Zn intake has adverse effects on the nervous, immune, skeletal and reproductive systems; influences the proliferation, pathogenesis and pathophysiology of some diseases (Jurowski et al. 2014), and their recovery time; and impairs mental capacity (Prasad 2013).

The world regions most markedly affected by Zn deficiency are those where the population's diet is based on cereals (rice, wheat and maize mainly), which are commonly developing countries (Cakmak 2008) in Central Asia, the Middle-East and South–East Asia, as well as others in Africa and South America (Alloway 2009). The grain Zn concentration of staple cereals is generally inadequate to meet humans nutritional requirements (3–20 mg d^{-1} depending on age and sex; Brown et al. 2004). For instance, the concentration of Zn in grain of wheat is typically 20–30 mg kg^{-1} but can be as low as 15 mg kg^{-1} (Erdal et al. 2002), and the minimum acceptable concentration for human nutrition is around or above 40 mg kg^{-1} (Cakmak and Kutman 2018). It is also important to note that wheat is considered to be a susceptible crop to Zn deficiency, hence, reducing grain quality and Zn concentration (Zou et al. 2012).

Zinc deficiency symptoms are observed in at least 49% of the world's cereal growing area (Graham and Welch 1997). Therefore, low grain Zn content of staple cereals is often associated to the soils they are grown in. The concentration and availability of Zn in the soil solution is mainly influenced by adsorption–desorption reactions (Catlett et al. 2002), which are conditioned by the mineralogy and chemical properties of the soil. According to Alloway (2009), the soil components and properties that significantly influence the concentration and availability of Zn in the soil solution are: a low total Zn content, a high soil pH, and high soil organic matter and calcite contents. Calcareous soils, which account for more than one-third of arable land worldwide (Chen and Barak 1982), induce deficiencies in different nutrients (including Zn). This is due to their alkaline pH (buffered at 7.5–8.5) that results from their high $CaCO_3$ content (Alloway 2009). Also, high P fertilizer rates and soil P contents are known to have adverse effects on soil Zn availability and plant uptake (Loneragan and Webb 1993; Zhang et al. 2015). In addition, a soil index that considers available P and Zn in soil has been suggested to predict the effect of Zn fertilization on yield of wheat plants in pots (Sacristán et al. 2019) but there is a lack of information under field conditions.

A number of strategies have been proposed to increase soil Zn availability and plant Zn uptake. Most are based on genetic (plant breeding) or agronomic biofortification (application of Zn fertilizers, intercropping, no tillage; Cakmak 2008; Xue et al. 2016). Zn fertilizers applied to the soil appear to be a reasonable choice for farmers in the short-term (Cakmak 2008) due to the reduced costs associated to this strategy. Within this, the application of optimum rates of Zn fertilizers to the soil improves soil Zn availability and crop yield but this option does not guarantee adequate grain Zn concentration (Cakmak 2008; Zou et al. 2012; Liu et al. 2017; Ahsin et al. 2020). Although soil application of Zn can also enhance grain Zn concentration, foliar applications are proved to be more effective for biofortification purposes (Cakmak 2008; Mabesa et al. 2013; Joy et al. 2015; Ram et al. 2016; El-Dahshouri et al. 2017; Zia et al. 2020). The effectiveness of foliar Zn spraying is enhanced when applied at late growth stages but this method cannot compete with soil application when considering yield increase (Zou et al. 2012; Cakmak and Kutman 2018; Ahsin et al. 2020). In addition, foliar spraying raises costs unless Zn fertilizer is included in farmers' foliar pesticidal treatments (Ram et al. 2016). Although grain yield and grain Zn concentration are inversely related (McDonald et al. 2008), some studies have revealed that both can be increased by using an appropriate Zn fertilization strategy (Zou et al. 2012; Chattha et al. 2017). Field experiments in other locations and involving different management systems, crop histories (rotations) and wheat cultivars are needed (Zou et al. 2012) to accurately identify effective strategies for increasing yields and/or biofortify wheat under different circumstances (e. g. climate, soils).

Soil nutrient limitations for plants are common in Spain and other countries in the Mediterranean region, which abound with calcareous soils. Moreover, there is a lack of field studies on the incidence of Zn deficiency and its effects on plant growth and yield in countries of southern Europe such as Spain, where deficiencies in other micronutrients have been widely studied (for example, iron; del Campillo and Torrent 1992; Ryan et al. 2012). Based on the foregoing, the main aims of this work were (i) to identify a soil indicator allowing one to predict the response in terms of yield of durum wheat grown in calcareous Vertisols under Mediterranean climate to Zn application to soil; (ii) to assess and compare the effect on wheat yield and biofortification of various Zn fertilization strategies, including combinations of two fertilization methods (soil application and Zn foliar spraying), different Zn rates (durum wheat) and different spraying times (bread wheat); and (iii) to assess the effect of Zn fertilization (soil VS. foliar applications) on crop P uptake and grain P concentration. For the first aim, nine field experiments were developed in six seasons (2012–2017), in which durum wheat was used. Two more field experiments were conducted to achieve the last two aims cited: to compare the effect of soil and foliar applications on plant growth, Zn and P uptake, one with durum wheat (2017–2018, in which different Zn rates were used) and another with bread wheat (2018–2019, in which different spraying times were considered). The starting hypotheses were (i) that durum wheat yield will be increased at least by the highest Zn rate applied to the soils with the lowest Zn availability and high P content; (ii) that the effectiveness of foliar spraying will depend on Zn rate (tested in durum wheat) or application time (wheat growth stage), being later applications more appropriate (tested in bread wheat); and (iii) that crop P uptake will not be affected by Zn fertilization but grain P concentration could be slightly affected by the highest rates of Zn applied to the soil and/or by foliar applications.

Material and methods

Study site and experimental design

Eleven field experiments were conducted on wheat crops from 2012 to 2019 in a farming area located at 37°47′56.9″ N, 4°32′54.1″ W in the province of Córdoba. The land in this area is cropped with wheat–sunflower/chickpea/rapeseed rotations. The area has a Mediterranean climate (*Csa* according to Köppen's classification) and had a mean annual precipitation of 614 mm over the period 2005–2019. Precipitation is highly variable (standard deviation 214 mm in this period) and concentrated in a few months each year (Fig. 1 and Table 1). The dominant soils in the area are Vertisols (IUSS Working Group WRB 2014) developed on marls. Due to their calcareous characteristics (a buffered pH of 8.0–8.5), limitations in P and some micronutrients such as Zn are to be expected.

Triticum durum from 2012 to 2018 and *Triticum aestivum* in the 2018–2019 season were grown in our field experiments (Table 1 provides additional details, including the cultivars used in each season). Our study was done in collaboration with *SAT Córdoba*, an organization that advises local farmers. Three different cultivars of durum wheat and one of bread wheat (in the last season) were used because the field experiments were performed in farms that belong to these farmers and the same plant material and management as that of the local farmer was adopted (except for Zn fertilization). Wheat was drilled between November and December, using a row spacing of 0.15 m after ploughing to a depth of 0.20 m, and harvested between May and June depending on the season. Crop management was similar in all the field experiments and additional details on fertilization, pesticides, herbicides, etc. for the 2017–2018 and 2018–2019 seasons are shown in Table S1.

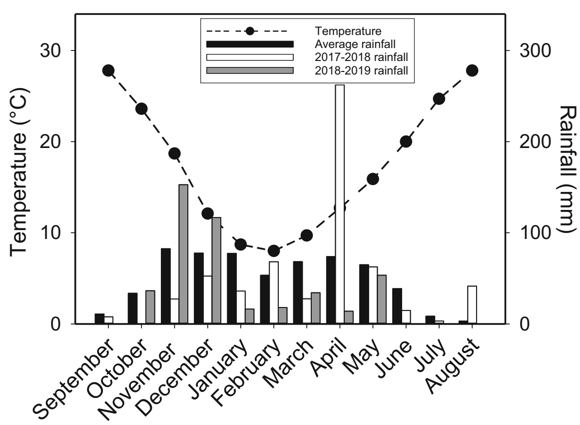

Fig. 1 Average monthly temperature and rainfall at Córdoba airport over the 2005–2019 period (temperature and average rainfall, respectively), and monthly rainfall at the same site in the 2017–2018 and the 2018–2019 seasons

Table 1 Summary of the eleven field experiments (2012–2019) developed in Córdoba (southern Spain) to test different soil and foliar applications (2017–2018 and 2018–2019 only) of Zn

Field	Season	Variety	Precipitation (Nov.-June)	P_{Olsen}	Zn_{DTPA}	P_{Olsen}/Zn_{DTPA}	Yield (t ha^{-1})					
			(mm)	(mg kg^{-1})	(mg kg^{-1})		Control (kg Zn ha^{-1}) 0	Soil application (kg Zn ha^{-1}) 0.3	3	6 / 10	Foliar spraying (kg Zn ha^{-1}) 0.64 B	1.28 SE – F-SE / F-F / F-SE/F
Field experiment												
Durum wheat												
ENC1	2012–2013	Calero	778	20.0	0.25	80.0	4.9 b	5.3 a	5.3 a			
ENC2	2013–2014	Calero	428	4.3	0.18	23.6	2.8	2.8	2.9			
TOR1	2013–2014	Carpio	428	4.6	0.22	20.9	3.6	3.4	3.5			
	2014–2015	Carpio	414	4.6	0.25	18.4	2.7	2.8	3.1			
TOR2	2014–2015	Carpio	413	8.5	0.50	17.0	3.8	3.7	3.5			
ENC3	2015–2016	Calero	504	9.8	0.16	61.3	0.6	0.5	0.5			
ORI	2015–2016	Calero	504	10.7	0.22	48.6	1.3	1.4	1.3			
ENC4	2016–2017	Amilcar	530	8.9	0.21	42.6	4.6	4.6	4.6			
PAR	2016–2017	Carpio	530	14.5	0.30	48.3	3.6	3.5	3.4			
ENC5	2017–2018	Carpio	551	18.2	0.46	39.6	6.6		6.5	7.0 / -	6.8	6.4
Bread wheat												
ENC6	2018–2019	Mulhacén	405	18.2	0.46	39.6	1557			- / 1.6		1.5 / 1.5 / 1.6

Different letters in yields indicate significant differences between the mean values of the treatments ($n = 4$) according to the posthoc LSD test ($p < 0.05$). Although some field experiments share the same name, a different number was used to indicate that each one was developed in a new area of the same farm and Zn treatments were only applied once to each field experiment. The last two experiments (2017–2018 and 2018–2019) were done to assess and compare the effect on wheat yield, biofortification and crop P uptake of various Zn fertilization strategies, while the rest were done to find a soil indicator to predict durum wheat yield response to soil Zn fertilization. Treatments: control or *0* (no Zn applied); *S3*, *S6* and *S10* (3, 6 and 10 kg Zn ha^{-1}, respectively), applied to the soil before sowing; *F0.64* and *F1.28* (0.64 or 1.28 Zn ha^{-1} applied by foliar spraying at early booting-B); *F-SE* (1.28 kg Zn ha^{-1} applied by foliar spraying at stem elongation); *F-F* (1.28 kg Zn ha^{-1} applied by foliar spraying at early flowering); *F-SE/F* [1.28 kg Zn ha^{-1} applied by foliar spraying at stem elongation (50%) and early flowering (50%)]. Not all the treatments were applied in each field experiment

P_{Olsen}: soil available P extracted with $NaHCO_3$; Zn_{DTPA}: soil available Zn extracted with DTPA (diethylenetriamine penta-acetic acid)

A completely randomized block design with four blocks (one replication per block, $n = 4$) was established at each site. Plot size was 11 × 3.2 m except in the 2018–2019 season, where these were of 9 × 3 m. Plots were separated by a 3 m wide untreated cultivated aisle in all experiments.

Zn treatments

Zn at different rates was applied to the soil before sowing (in all experiments) or sprayed to leaves (two last seasons, 2017–2018 and 2018–2019). Zn sulphate heptahydrate ($ZnSO_4 \cdot 7H_2O$) was used in all cases. A dissolution of $ZnSO_4 \cdot 7H_2O$ (1.070 g Zn L^{-1} except for the lowest rate in 2017–2018, which was 0.535 g Zn L^{-1}) was prepared for each foliar treatment. The dissolutions were applied at 1200 L ha^{-1} with a manual backpack sprayer connected to a boom sprayer with four nozzles at different wheat phenological stages (Zadoks et al. 1974), depending on the particular season. No leaf damage was observed upon application of the foliar treatments. Fig. S1 shows the schedule of the experiments included in this study (season, crop, variety, and Zn treatments used in each field experiment). Note that each field experiment was developed during one season. Although some field experiments were carried out in the same field site (Table 1), a different area was used for each one. Therefore, Zn treatments were not repeated in the same area in different field experiments.

Soil application: 2012–2013 to 2016–2017 seasons (durum wheat)

These experiments were used to establish the critical value of the soil available P/available Zn ratio in Vertisols under Mediterranean climate for durum wheat crops triggering a yield response upon the application of Zn to the soil. For this purpose, three Zn treatments were used in 9 field experiments conducted from 2012 to 2017 (upper part of Fig. S1), namely: *0* (Control; no Zn applied; 0 kg Zn ha^{-1}); *S0.3* (0.3 kg Zn ha^{-1} applied to the soil) and *S3* (3 kg Zn ha^{-1} applied to the soil). Yield data of the seasons detailed along with the wheat yield data of season 2017–2018 (*0* and *S3* only) were used for this part of the study.

Soil and foliar applications: 2017–2018 (durum wheat) and 2018–2019 (bread wheat) seasons.

Two field experiments were performed to assess and compare the effect of various Zn fertilization strategies on wheat yield, crop biofortification and P uptake. The Zn treatments included two methods (soil application and foliar Zn spraying), variable Zn rates (durum wheat, 2017–2018) and different application times of the foliar treatments (bread wheat, 2018–2019; bottom part of Fig. S1). The treatments for the experiment conducted in 2017–2018 were as follows: *0* (Control; no Zn applied; 0 kg Zn ha^{-1}); *S3* (3 kg Zn ha^{-1} applied to the soil); *S6* (6 kg Zn ha^{-1} applied to the soil); *F0.64* [0.64 kg Zn ha^{-1} sprayed to plants at early booting (viz., 41-flag leaf sheath extending, April 4)]; and *F1.28* [1.28 kg Zn ha^{-1} sprayed to plants at early booting stage (viz., 41-flag leaf sheath extending, April 4)]. This experiment (2017–2018, durum wheat) aimed at comparing the effect on yield and grain Zn concentration for the different Zn application strategies detailed above.

The experiment performed in the 2018–2019 season (bread wheat) included the following Zn treatments: *0* (Control; no Zn applied; 0 kg Zn ha^{-1}); *S10* (10 kg Zn ha^{-1} applied to the soil); *F-SE* [1.28 kg Zn ha^{-1} sprayed to plants at the stem elongation stage (33-third node detectable, March 20)]; *F-F* [1.28 kg Zn ha^{-1} sprayed to plants at the flowering stage (62-early flowering, April 15)]; and *F-SE/F* [(1.28 kg Zn ha^{-1} sprayed to plants at the stem elongation (33-third node detectable, 50% of the total amount of Zn, March 20) and flowering stage (62-early flowering, 50% of the total amount of Zn, April 15)]. This experiment (2018–2019, bread wheat) was intended to compare the effect of Zn foliar spraying at two different growth stages, including the effect of splitting the amount of Zn between these two growth stages, with those of applying Zn to the soil and using no Zn fertilizer.

Soil sampling and analysis

Four representative soil samples from each field were collected down to 0–0.2 m at the beginning of each season and sieved to 1 cm to remove roots. Then, 0.5 kg of each sample was sieved to 2 mm in the laboratory prior to analysis. After dispersion with sodium hexametaphosphate, soil texture was determined with the pipette method (Gee and Bauder 1986) and organic carbon (OC) by rapid dichromate oxidation (Walkley and Black 1934). Calcium carbonate equivalent was estimated according to van Wesemael (1955). Soil pH was determined potentiometrically in a 1:2.5 soil:water suspension (pH meter GLP 21, Crison Instruments SA) and electrical conductivity (EC) of the 1:5 (*w*/*v*) soil:water suspension with a conductivity meter (micro CM 2200, Crison Instruments SA). Available soil P (P_{Olsen}) was extracted according to Olsen et al. (1954) and measured by using the Molybdate Blue method (Murphy and Riley 1962), and labile Zn in soil (Zn_{DTPA}) was extracted with diethylenetriaminepentaacetic acid (DTPA) at 25 °C (1:2 soil/DTPA suspension; Lindsay and Norvell 1978) and measured by atomic absorption spectrophotometry. All the analyses were done for the soil collected from the field experiments developed in 2017–2018 (ENC5) and 2018–2019 (ENC6) but only P_{Olsen} and Zn_{DTPA} in the rest of the soil samples collected from the other seasons. However, the 11 field experiments were developed in the same farming area.

Plant sampling, plant production and yield

In the first 9 field experiments (2012–2017), a Hege 140 small-plot combine harvester was used to cut wheat plants 15 cm above the ground in a 16.5 m^2 area in each plot. Grain yield was determined by weighing the samples after drying them in an oven at 60 °C for 72 h. In the 2017–2018 and 2018–2019 seasons, plants at different phenological stages were harvested using mechanical scissors and cutting the plant material 2 cm above the ground. Plants were sampled from two 0.4 × 0.4 m metal frames a total of 7 times the former season and 5 the latter (Zadoks et al. 1974; Table 2). These plants were generously washed in the laboratory with tap water (30 s) and then with deionized water (30 s, twice) to remove the excess of Zn from the surface, not taken up after foliar spraying. Then, straw, spike and/or grain were isolated when possible and weighed after drying at 60 °C for at least 72 h. The total amount of biomass was calculated as the combination of those the different plant parts for each sampling time, Zn treatment and season. Next, the samples were ground in a mill and a subsample of each plant part (0.2 g) was digested with 3 mL of 65% nitric acid and 1 mL of 60% perchloric acid (Zasoski and Burau 1977). The P and Zn contents of the different tissues from plants harvested in the 2017–2018 (durum wheat) and 2018–2019 (bread wheat) seasons were determined by using the Molybdate Blue method (Murphy and Riley 1962) and by atomic absorption spectrophotometry, respectively (Fig. S1).

Phosphorus and zinc uptake, zinc use efficiency and zinc gain relative to non-zinc fertilized plants

Phosphorus and Zn uptake by grain and crop at harvest in the 2017–2018 and 2018–2019 seasons (Fig. S1) were calculated by multiplying the P and Zn concentrations of each plant part by their amount of dry matter and adding them up. The Zn use efficiency (ZnUE) of grains and crops (grain and straw) were calculated from Eqs. 1 and 2:

$$ZnUE, grain = \frac{Zn, gt - Zn, gc}{Zn\ applied} \times 100 \quad (1)$$

$$ZnUE, crop = \frac{Zn, ct - Zn, cc}{Zn\ applied} \times 100 \quad (2)$$

where *Zn,gt* and *Zn,ct* are the grain and crop Zn uptake, respectively, from each of the Zn treatment plots (*S3*, *S6*, *S10*, *F0.64*, *F1.28*, *F-SE*, *F-F* or *F-SE/F*); *Zn,gc* and *Zn,cc* are the mean grain and crop Zn uptake from the Control plots; and *Zn applied* is the amount of Zn fertilizer applied to the plots with each Zn treatment.

Zn grain gain and Zn crop gain relative the Control plots (0 kg Zn ha^{-1}) were calculated from Eqs. 3 and 4:

$$Zn\ grain\ gain = \frac{Zn, gt - Zn, gc}{Zn, gc} \times 100 \quad (3)$$

$$Zn\ crop\ gain = \frac{Zn, ct - Zn, cc}{Zn, cc} \times 100 \quad (4)$$

All variables in the previous four equations are expressed in g Zn ha^{-1} —by exception, *Zn applied* is in kg Zn ha^{-1}.

Statistical analysis

The durum wheat yield results of each season (from 2012 to 2018) were analysed following a one-way analysis of variance (ANOVA) in order to identify significant differences between non-Zn fertilized plots and plots fertilized with variable Zn rates (viz., 0.3 and 3 kg Zn ha^{-1} from 2012 to 2017, and 3 and 6 kg Zn ha^{-1} in 2017–2018 season). Biomass, and P and Zn concentrations of each plant part, in the 2017–2018 (durum wheat) and 2018–2019 (bread wheat) seasons were analysed by repeated measures ANOVA (RM ANOVA; sampling time and Zn treatment) with a

Table 2 Sampling times for the 2017–2018 (durum wheat) and 2018–2019 (bread wheat) seasons

Season	Date	DAS	Growth stage		Analyses
2017–2018	Durum wheat				
1	15th February	86	21	Tillering – Main steam and one tiller	Biomass, [P] and [Zn]
2	13th March	112	34	Stem elongation – Fourth node detectable	Biomass, [P] and [Zn]
3	20th March	119	37	Stem elongation – Flag leaf just visible	Biomass, [P] and [Zn]
4	4th April	134	41	Booting – Flag leaf sheath extending	Biomass, [P] and [Zn]
5	17th April	147	59	Ear emergence from boot – Emergence completed	Biomass, [P] and [Zn]
6	14th May	174	79	Milk development – Very late milk, half-solid/half-liquid	Biomass, [P] and [Zn]
7	20th June	221	94	Ripening – Grain loosening in daytime	Biomass, yield, [P], [Zn]
2018–2019	Bread wheat				
1	11th February	61	12	Leaves on main shoot – Second leaf more than half visible	Biomass, [P] and [Zn]
2	26th March	104	38	Booting – Flag leaf and collar visible	Biomass, [P] and [Zn]
3	30th April	138	70.5	Kernel extending – Kernels extended 50%	Biomass, [P] and [Zn]
4	23rd May	161	93	Ripening – Kernels loosening in daytime	Biomass, [P] and [Zn]
5	27th May	165	94	Ripening – Grain loosening in daytime	Yield, [P], [Zn]

[a] DAS days after sowing

blocking factor. When the sampling time × Zn treatment interaction was significant ($P < 0.05$), and with all other variables (P and Zn concentrations, P and Zn grain and crop uptake, ZnUE-grain, ZnUE-crop, Zn grain gain and Zn crop gain), the results were subjected to one-way ANOVA (Zn treatment). When the one-way ANOVA was significant, the LSD post-hoc test was used to identify significant differences between Zn treatments, after checking for variance homoscedasticity with Levene's test, and logarithmic or square-root transformation of the data if needed. The variables yield (durum wheat), annual rainfall, cumulative rainfall from February to April each season, P_{Olsen}, Zn_{DTPA} and the P_{Olsen}/Zn_{DTPA} ratio (including the data from 2012 to 2018) were subjected to linear regression analysis. All statistical analyses were done with the software Statistix v. 10.0 from Analytical Software (Tallahassee, FL, USA).

Results

Soil analysis

The eleven field experiments were developed in calcareous Vertisols on marls and so the properties of the soils of each experiment were similar. These soils have a high content in clay but a low content in organic carbon (OC < 10 g kg^{-1}; Table 3). By virtue of its high carbonate content, the studied soils had a buffered pH of 8.0–8.5 and the low electrical conductivity of their water extract posed no salinity-related problems (Table 3). P_{Olsen} ranged from 4 to 20 mg kg^{-1} and Zn_{DTPA} from 0.16 to 0.50 mg kg^{-1}, which resulted in a wide soil P_{Olsen}/Zn_{DTPA} ratio (17–80; Tables 1 and 3).

Table 3 Soil properties of the field in which wheat was grown in the 2017–2018 and 2018–2019 seasons (mean ± standard error, $n = 4$)

Soil properties	Units	
Clay	g kg^{-1}	440±4
Silt	g kg^{-1}	264±8
Sand	g kg^{-1}	320±37
OC	g kg^{-1}	6.5±0.5
$CaCO_3$	g kg^{-1}	410±6
$pH_{1:2:5}$		8.1±0.1
$EC_{1:5}$	dS m^{-1}	0.18±0.01
P_{Olsen}	mg kg^{-1}	18.2±1.6
Zn_{DTPA}	mg kg^{-1}	0.46±0.03
Soil P_{Olsen}/Zn_{DTPA}		39.6

OC: organic carbon; $CaCO_3$: calcium carbonate content; $pH_{1:2.5}$: soil pH in the 1:2.5 soil:water extract; $EC_{1:5}$: electrical conductivity of the 1:5 soil:water extract; P_{Olsen}: soil available P extracted with $NaHCO_3$; Zn_{DTPA}: soil available Zn extracted with DTPA (diethylenetriamine pentaacetic acid)

Biomass time course and yield at harvest

Wheat yield significantly increased in only 1 of the field experiments conducted (viz., ENC1, 2012–2013 season), where the application of 0.3 and 3 kg Zn ha^{-1} raised yield to 5.33 and 5.25 t ha^{-1}, respectively, compared to 4.68 t ha^{-1} in the non-Zn fertilized soil (Table 1). The variance in wheat yield of the non-Zn fertilized plots was explained by cumulative rainfall from February and April —both months included—, with $R^2 = 0.46$, $p = 0.022$ (Fig. S2A), but not by any other measured variables (annual rainfall, P_{Olsen}, Zn_{DTPA} or soil P_{Olsen}/Zn_{DTPA} ratio; Figs. S2B–S2D and Table 1).

In the last two seasons, wheat biomass for the 2017–2018 (durum wheat) and 2018–2019 (bread wheat) seasons was not influenced by any of the Zn treatments assayed ($p = 0.589$ and $p = 0.434$, respectively for each season; Fig. 2). This was also the case for yield at harvest ($p = 0.509$ for the 2017–2018 season and $p = 0.489$ for the 2018–2019 season; Fig. 3). As expected, however, biomass significantly increased with time in both seasons ($p < 0.001$; Fig. 2 and Table S2). The sampling time × Zn treatment interaction was not significant in either season ($p = 0.913$ and $p = 0.893$, respectively; Table S2).

Phosphorus and zinc concentrations in shoot and grain

The sampling time × Zn treatment interaction for shoot P concentration was significant in the 2017–2018 season (durum wheat, $p = 0.006$) but not in the 2018–2019 season (bread wheat, $p = 0.075$; Table S2). The P concentration of shoots increased from the earlier phenological stages to stem elongation and early booting before decreasing in ripening (Fig. 4A and B). No significant differences in shoot P concentration were found between Zn fertilization methods (soil application or foliar spraying) in any of the two last seasons.

Also, a significant time × Zn treatment interaction was found for Zn shoot concentrations both in durum wheat and bread wheat ($p < 0.001$ in both crops, 2017–2018 and 2018–2019, respectively; Table S2). The effect of Zn fertilization was apparent from the end of heading (2017–2018; growth stage 59) and kernel extending (2018–2019, growth stage 71) to ripening, but mainly in the Zn-sprayed plants. Thus, treatment *F0.64* and, especially, *F1*.28, significantly increased shoot Zn concentrations in durum wheat (2017–2018 season; growth stages 59, 79 and 94; Fig. 4C). Foliar Zn spraying at flowering (*F-F*) and fertilizer splitting between stem elongation and flowering (*F-ES/F*), were the most efficient treatments in bread wheat (2018–2019 season; growth stages 71 and 93; Fig. 4D), followed by foliar spraying at stem elongation (*F-SE*) and application to the soil (*S10*).

Although grain P concentration was not altered in durum wheat (2017–2018, Fig. 5A) or bread wheat (2018–2019, Fig. 5B), that of Zn at harvest was significantly influenced by Zn fertilization. Moreover, Zn concentration of grain decreased following these sequences: foliar spraying of 1.28 and 0.64 kg Zn ha^{-1} at early boot stage, soil applications (6 and 3 kg Zn ha^{-1}), and finally, non-fertilized durum wheat ($F1.28 > F0.64 \geq S6 \geq S3 = 0$) in 2017–2018 (Fig. 5C); and foliar spraying at flowering, followed by foliar spraying split between at stem elongation and flowering, then foliar spraying at stem elongation, soil application (10 kg Zn ha^{-1}) and, finally, non-fertilized bread wheat ($F\text{-}F > F\text{-}SE/F > F\text{-}SE \geq S10 \geq 0$) in 2018–2019 (Fig. 5D). Regarding grain P:Zn ratio (Table 4), no significant differences among Zn treatments were found in durum wheat in the 2017–2018 season ($p = 0.639$). On the other hand, the Zn treatments significantly reduced the ratio relative to non-Zn fertilized plots in bread wheat in the 2018–2019 season ($p < 0.001$). This was especially so with foliar spraying at the flowering stage, followed by splitting of sprayed Zn between stem elongation and flowering, and foliar application at stem elongation only (Table 4). Application of Zn to the soil (treatment *S10*) reduced the ratio, albeit not significantly, relative to non-Zn fertilized plots.

Phosphorus and zinc uptake

Grain and crop P uptake were not affected by Zn fertilization in either season (durum wheat in 2017–2018 and bread wheat in 2018–2019), while grain and crop Zn uptake significantly increased —except for grain Zn uptake in the 2017–2018 season (Table 4). None of the soil treatments (*S3* and *S6* for durum wheat and *S10* for bread wheat) significantly increased these two variables relative to the non-Zn fertilized plots in each season. However, Zn foliar spraying did increase Zn uptake, mainly at the highest Zn rate in the 2017–2018 season (treatment *F1.28*, durum wheat) and foliar Zn spraying at the flowering stage only (*F-F*), followed by

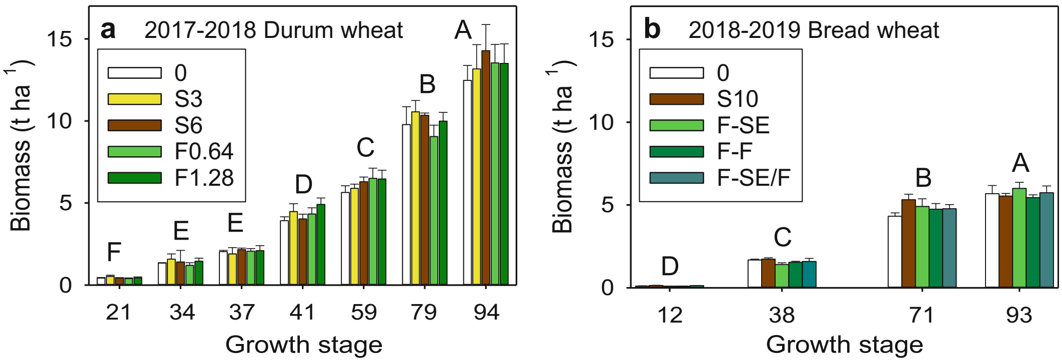

Fig. 2 Wheat biomass as a function of sampling time (Zadoks scale) and Zn treatment (mean ± standard error, $n = 4$ per Zn treatment) in the 2017–2018 (durum wheat) and 2018–2019 (bread wheat) seasons. Different letters indicate significant differences between the mean value for each sampling time as per the LSD test at $p < 0.05$. Treatments: *0* (no Zn applied); *S3*, *S6* and *S10* (3, 6 and 10 kg Zn ha^{-1}, respectively), applied to the soil before sowing; *F0.64* and *F1.28* (0.64 or 1.28 Zn ha^{-1} applied by foliar spraying at early booting); *F-SE* (1.28 kg Zn ha^{-1} applied by foliar spraying at stem elongation); *F-F* (1.28 kg Zn ha^{-1} applied by foliar spraying at early flowering); *F-SE/F* [1.28 kg Zn ha^{-1} applied by foliar spraying at stem elongation (50%) and early flowering (50%)]

foliar spraying split between stem elongation and flowering (*F-SE/F*) in the 2018–2019 season (bread wheat).

Zinc use efficiency and zinc gain

Grain ZnUE was smaller than 5% in the 2017–2018 season (durum wheat), with no significant differences between soil (0.1% with *S3* and 0.4% with *S6*) or foliar treatments (4.6% with *F0.64* and 3.8% with *F1.28*, $p = 0.126$). Also, grain ZnUE was smaller than 1.6% in the 2018–2019 season (bread wheat), having significant differences between Zn treatments; higher grain ZnUE in foliar treatments (viz., 1.5% with *F-F*, 1.3% with *F-ES/F*, 0.5% with *F-ES*) were observed in comparison with soil treatment (0.1% with *S10*; $P < 0.001$; Table 4). Crop ZnUE exhibited a similar variation pattern and amounted to 0.2–1.3% in durum wheat and 0.1% in bread wheat with the soil treatments and 16.2–19.0% in durum wheat and 3.1–9.4% in bread wheat with the foliar treatments, being the differences between the two significant ($p < 0.001$ for both seasons; Table 4).

Crop Zn gain relative to non-Zn fertilized plots differed significantly between treatments in both the 2017–2018 (durum wheat, $p = 0.035$) and 2018–2019 season (bread wheat, $p < 0.001$). The largest gains were

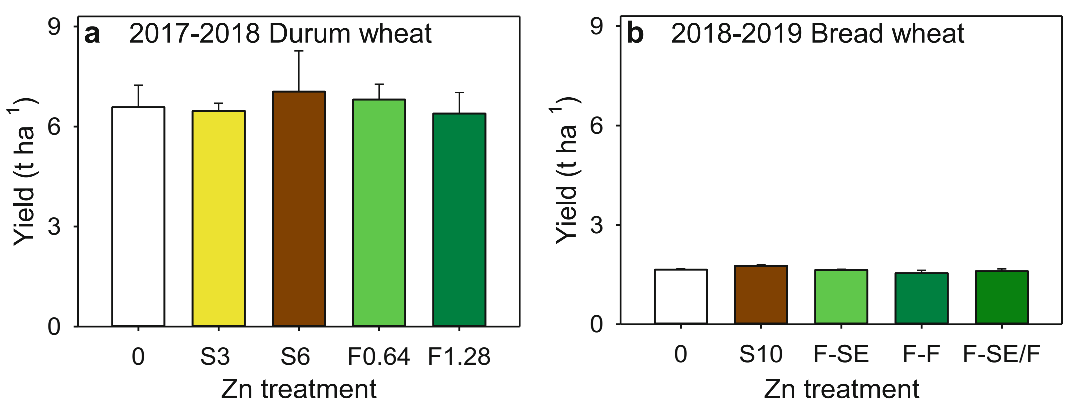

Fig. 3 Grain yield of wheat plants at harvest as a function of Zn treatment (mean ± standard error, $n = 4$ per Zn treatment) in the 2017–2018 (durum wheat) and 2018–2019 (bread wheat) seasons. Treatments: *0* (no Zn applied); *S3*, *S6* and *S10* (3, 6 and 10 kg Zn ha^{-1}, respectively), applied to the soil before sowing; *F0.64* and *F1.28* (0.64 or 1.28 Zn ha^{-1} applied by foliar spraying at early booting); *F-SE* (1.28 kg Zn ha^{-1} applied by foliar spraying at stem elongation); *F-F* (1.28 kg Zn ha^{-1} applied by foliar spraying at early flowering); *F-SE/F* [1.28 kg Zn ha^{-1} applied by foliar spraying at stem elongation (50%) and early flowering (50%)]

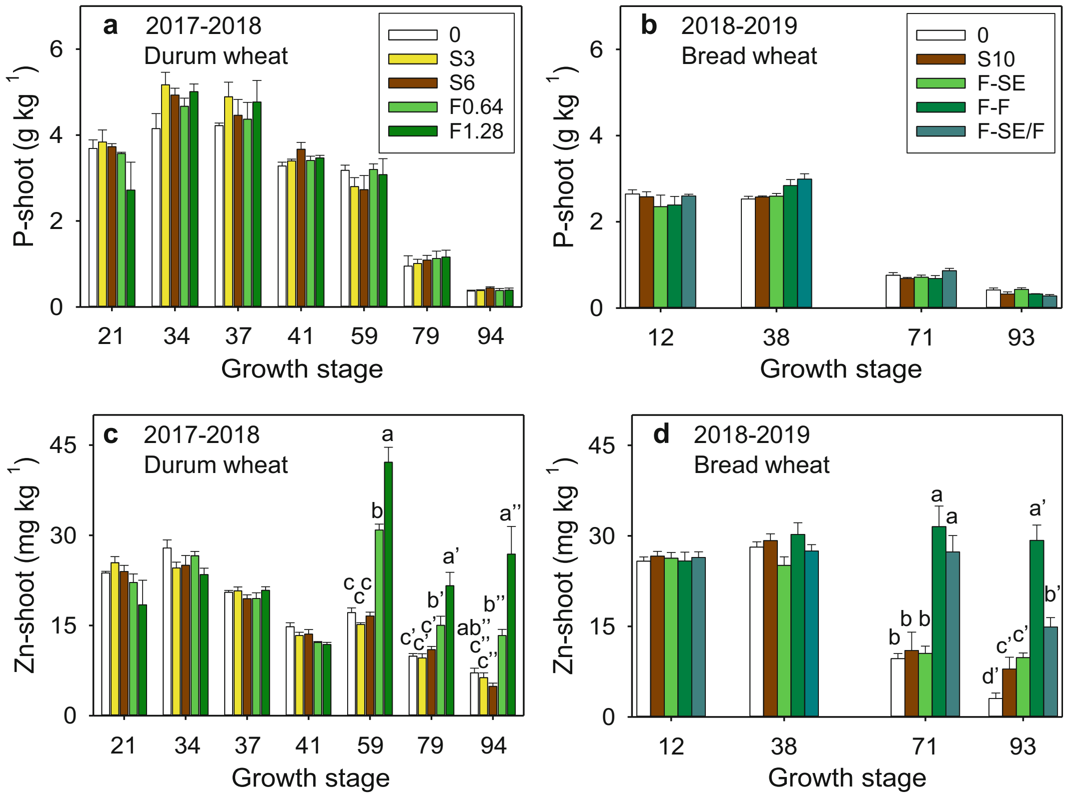

Fig. 4 Shoot P and Zn concentrations of wheat plants as a function of sampling time (Zadoks scale) and Zn treatment (mean ± standard error, $n = 4$ per Zn treatment) in the 2017–2018 (durum wheat) and 2018–2019 (bread wheat) seasons. Different letters indicate significant differences between means for the different Zn treatments (lowercase letters) as per the LSD test at $p < 0.05$. Treatments: *0* (no Zn applied); *S3*, *S6* and *S10* (3, 6 and 10 kg Zn ha^{-1}, respectively), applied to the soil before sowing; *F0.64* and *F1.28* (0.64 or 1.28 Zn ha^{-1} applied by foliar spraying at early booting); *F-SE* (1.28 kg Zn ha^{-1} applied by foliar spraying at stem elongation); *F-F* (1.28 kg Zn ha^{-1} applied by foliar spraying at early flowering); *F-SE/F* [1.28 kg Zn ha^{-1} applied by foliar spraying at stem elongation (50%) and early flowering (50%)]

achieved with foliar treatments, specifically in the highest rate (*F1.28,* 24.8%) in durum wheat and in the application of Zn at flowering stage (*F-F*, 43.7%) and the split between flowering stage and stem elongation (*F-SE/F*, 37.1%) in bread wheat (Table 4). Although differences in grain Zn gain were not significant in the 2017–2018 (durum wheat, $p = 0.623$) or 2018–2019 season (bread wheat, $p = 0.140$), they exhibited the same trend as crop Zn gain.

Discussion

Effect of Zn treatments on wheat biomass and yield

Cumulative rainfall from February to April was the most influential factor limiting the yield of durum wheat, which ranged from 0.59 t ha^{-1} with 132 mm of rainfall to 6.58 t ha^{-1} with 358 mm. Water availability during this period is not only highly variable under the typical Mediterranean climate of the area but also, as shown here, crucial for wheat growth and yield. Thus, a low cumulative rainfall from February to April may have minimized the effects of Zn fertilization. Yield was apparently not influenced by any other soil measured variables (P_{Olsen}, Zn_{DTPA} or P_{Olsen}/Zn_{DTPA} ratio). It is also important to point out that the use of different durum wheat cultivars in our field experiments is another source of variation that may have affected yield (Fig. S2A).

One of the objectives of this study was to find a soil indicator to predict the response in terms of yield of durum wheat grown in calcareous Vertisols (under Mediterranean climate) to Zn application to the soil (first set of field experiments, developed in 2012–2018). Although the studied soils ranged widely in P_{Olsen} (4.3–

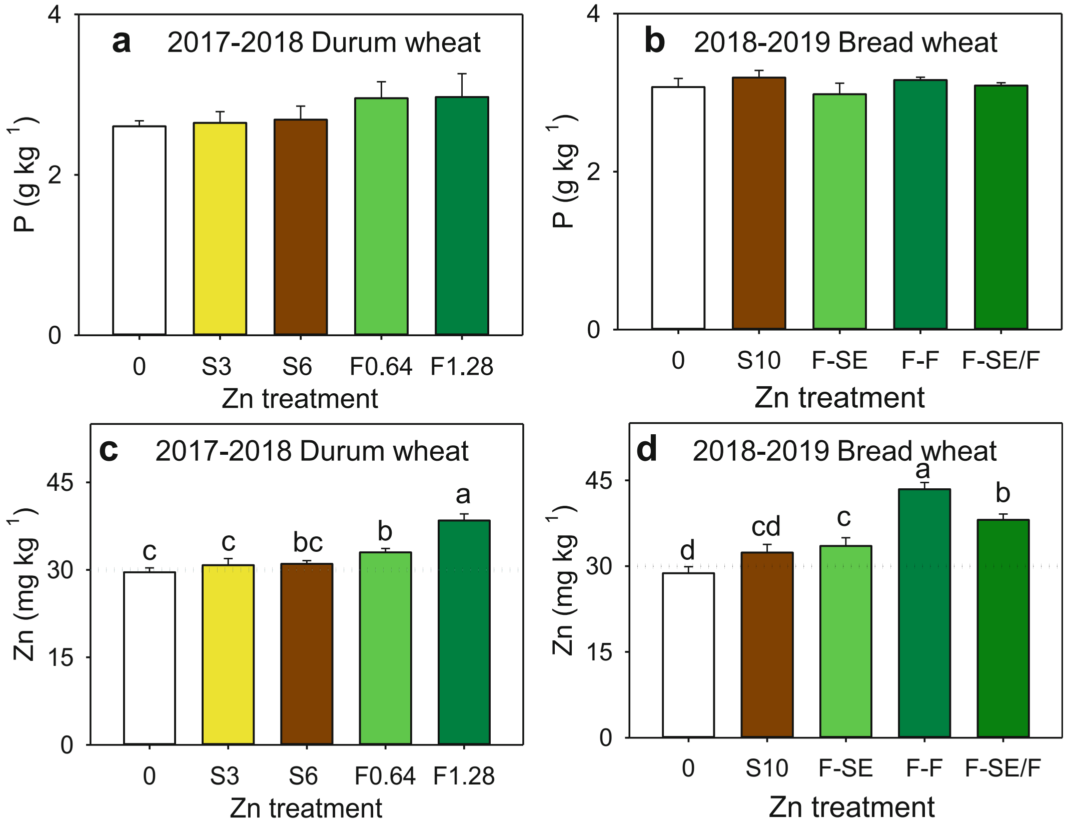

Fig. 5 Grain P and Zn concentrations of wheat plants at harvest as a function of Zn treatment (mean ± standard error, $n = 4$ per Zn treatment) in the 2017–2018 (durum wheat) and 2018–2019 (bread wheat) seasons. Different letters indicate significant differences between means for the different Zn treatments as per the LSD test at $p < 0.05$. The dotted line represents the limit for Zn deficiency in grain. Treatments: *0* (no Zn applied); *S3*, *S6* and *S10* (3, 6 and 10 kg Zn ha^{-1}, respectively), applied to the soil before sowing; *F0.64* and *F1.28* (0.64 or 1.28 Zn ha^{-1} applied by foliar spraying at early booting); *F-SE* (1.28 kg Zn ha^{-1} applied by foliar spraying at stem elongation); *F-F* (1.28 kg Zn ha^{-1} applied by foliar spraying at early flowering); *F-SE/F* [1.28 kg Zn ha^{-1} applied by foliar spraying at stem elongation (50%) and early flowering (50%)]

20.0 mg kg^{-1}) and P_{Olsen}/Zn_{DTPA} ratio (17–80), a critical available P/available Zn ratio was not found for durum wheat. Fertilizing the soil with Zn at 0.3 or 3 kg Zn ha^{-1} had no effect on yield relative to the non-Zn fertilized plots; except in the field experiment of the 2012–2013 season (ENC1), in which cumulative rainfall from February to April and the soil P_{Olsen}/Zn_{DTPA} ratio were the highest among the eleven field experiments (415.6 mm and 80, respectively). These results are in line with those of a previous study that revealed a positive effect of Zn fertilization on yield of pot-grown wheat plants with $P_{Olsen}/Zn_{DTPA} > 50$ (Sacristán et al. 2019). Two locations in our field experiments had a P_{Olsen}/Zn_{DTPA} ratio above 50 and one of them gave the lowest yields (0.59 t ha^{-1}; ENC3, 2015–2016). This can be attributed to the low cumulative rainfall from February to April (132 mm in this critical period for yield) registered in the cited experimental area. These results led us to hypothesize that limitations in space and soil can lead to smaller ratios for plants in pots (Sacristán et al. 2019) than those growing in the field.

Because the available Zn content (Zn_{DTPA}) of our soils was below the critical threshold for cereals proposed by Lindsay and Norvell (1978) (0.5 mg kg^{-1}), Zn fertilization was expected to increase wheat yield. Furthermore, seven of the studied locations had a rather low Zn_{DTPA} content (< 0.25 mg kg^{-1}). This was also the case with the values reported by Cakmak et al. (1996) for calcareous soils from Central Anatolia (Turkey), where application of Zn raised wheat yield. However, wheat yield was only increased in one of eleven field experiments involving Zn application to the soil conducted from 2012 to 2019 (viz., one using 0.3 and 3 kg Zn ha^{-1} in season 2012–2013, with $Zn_{DTPA} = 0.25$ mg kg^{-1}).

Table 4 Grain and crop P and Zn uptake, grain P:Zn ratio, grain and crop zinc use efficiency (ZnUE-grain and ZnUE-crop, respectively), and Zn grain and crop gain relative to the Control treatment (no Zn added; mean ± standard error, $n = 4$) in the 2017–2018 (durum wheat) and 2018–2019 (bread wheat) seasons

Zn treatment	Grain P uptake	Crop P uptake	Grain Zn uptake	Crop Zn uptake	Grain P:Zn ratio	ZnUE-grain	ZnUE-crop	Zn grain gain	Zn crop gain
Season	(kg ha^{-1})	(kg ha^{-1})	(g ha^{-1})	(g ha^{-1})		(% of applied Zn)	(% of applied Zn)	(% of applied Zn)	(% of applied Zn)
2017–2018	Durum wheat								
0	17.2±2.0	20.1±2.2	195±22	247±21 c	88±3	na	na	na	na
S3	17.1±0.7	20.6±0.9	200±11	254±11 c	87±7	0.1±0.4	0.2±0.4 b	2.2±5.8	23.6±4.6 b
S6	19.4±4.3	23.5±4.8	219±39	271±37 bc	87±7	0.4±0.6	1.3±0.6 b	12.3±19.8	30.8±15.0 b
F0.64	20.3±2.1	23.4±2.4	225±17	317±14 b	90±6	4.6±2.6	19.0±2.1 a	15.2±8.6	49.3±5.5 ab
F1.28	19.5±4.0	23.0±4.7	244±16	403±8 a	78±10	3.8±1.3	16.2±0.6 a	24.8±8.4	84.3±3.2 a
p value	0.835	0.843	0.417	**<0.001**	0.639	0.126	**<0.001**	0.623	**0.035**
2018–2019	Bread wheat								
0	4.8±0.2	5.6±0.1	45±2 c	96±16 c	107±7 a	na	na	na	na
S10	5.3±0.3	5.9±0.3	53±4 abc	110±11 c	99±3 ab	0.1±0.0 c	0.1±0.1 c	19.0±8.1	14.0±11.4 c
F-SE	4.6±0.4	5.6±0.3	51±3 bc	136±17 bc	89±5 bc	0.5±0.3 bc	3.1±1.3 c	14.5±7.6	41.2±17.7 bc
F-F	4.6±0.3	5.3±0.3	64±5.4 a	217±10 a	72±1 d	1.5±0.4 a	9.4±0.8 a	43.7±12.0	125.5±10.7 a
F-SE/F	5.0±0.3	5.5±0.3	61±4 ab	175±14 b	81±2 cd	1.3±0.3 ab	6.2±1.1 b	37.1±9.6	82.2±15.1 b
p value	0.496	0.6068	**0.019**	**<0.001**	**<0.001**	**0.017**	**<0.001**	0.140	**<0.001**

p value is the probability of the one-way ANOVA and different letters indicate significant differences between means for the different Zn treatments as per the LSD test at $p < 0.05$

Treatments: *0* (no Zn applied); *S3*, *S6* and *S10* (3, 6 and 10 kg Zn ha^{-1} , respectively), applied to the soil before sowing; *F0.64* and *F1.28* (0.64 or 1.28 Zn ha^{-1} applied by foliar spraying at early booting); *F-SE* (1.28 kg Zn ha^{-1} applied by foliar spraying at stem elongation); *F-F* (1.28 kg Zn ha^{-1} applied by foliar spraying at early flowering); *F-SE/F* [1.28 kg Zn ha^{-1} applied by foliar spraying at stem elongation (50%) and early flowering (50%)]

na: non-available

Besides that, wheat yield was never enhanced with foliar spraying. In addition, durum and bread wheat biomass in the 2017–2018 and the 2018–2019 seasons, respectively, was not altered by neither Zn treatment (soil application or foliar spraying), Zn rate (durum wheat) or time of foliar spraying (bread wheat). Sánchez-Rodríguez et al. (2017) found available Zn to affect neither wheat nor barley yield, but available soil P influenced yields of the two cereals grown on calcareous soils with a limited Zn availability. The lack of effect of Zn fertilization on yield should be related to the low Zn rate applied to the soil in our experiments, independently on the initial available P and labile Zn in soil of the different field experiments. In this sense, Liu et al. (2019) obtained significantly increased wheat yields with soil applications of 22.7 or 34.1 kg Zn ha^{-1} but not with 2.3, 5.7 or 11.4 kg Zn ha^{-1} (similar rates as the used in our study).

Wheat biofortification: Zinc rate (soil and foliar applications) and timing of foliar treatments

Another objective of this study was to assess the effect of Zn fertilization on crop P uptake and grain P concentration. None of the Zn treatments examined in the 2017–2018 (durum wheat) or 2018–2019 (bread wheat) seasons altered the P concentration of wheat shoot or grain, nor P uptake by grain or crop. To our knowledge, Zn fertilization has never been found to have an adverse effect on P uptake under field conditions. Nevertheless, the high available P/available Zn ratio in these calcareous Vertisols (17–80) may have reduced Zn availability or plant uptake, as found in previous experiments on similar soils under controlled conditions (Sánchez-Rodríguez et al. 2017; Sacristán et al. 2019).

With regard to Zn accumulation in shoots, only in bread wheat, Zn applied to the soil at a rate of 10 kg ha^{-1} (2018–2019) significantly increased Zn shoot concentration relative to the Control treatment. The other Zn applications to the soil assayed (3 and 6 kg ha^{-1}, 2017–2018) had no significant effect in Zn shoot concentration in durum wheat. The effect of the foliar Zn treatments resulted in an increase in grain Zn concentrations relative to non-Zn fertilized plots in durum and bread wheat (2017–2018 and 2018–2019, respectively). One of the reasons why foliar treatments were more efficient in increasing Zn concentration in shoots than soil application was that foliar spraying avoids the problems derived from the complex dynamics of Zn in calcareous soils. In fact, once Zn is applied to a calcareous soil, it is rapidly adsorbed onto the reactive surfaces of some soil components (Fe oxides, carbonates, clay minerals) and becomes largely unavailable to plants (Alloway 2008). Our Zn grain contents for the 2017–2018 and 2018–2019 seasons are consistent with those of Zou et al. (2019). These authors observed no effect on wheat yield but found an increase in Zn grain concentrations by effect of Zn foliar application, in experiments on calcareous soils from six different countries (China, India, Mexico, Pakistan, South Africa and Turkey).

With regard to Zn biofortification, soil application increased Zn grain concentration relative to non-Zn fertilized plots by 4.4% with 6 kg Zn ha^{-1} in the 2017–2018 season (durum wheat) and by 12.5% with 10 kg Zn ha^{-1} in the 2018–2019 season (bread wheat). However, the largest grain Zn concentration obtained with these treatments in durum and bread wheat was around 33.0 mg kg^{-1} and only slightly larger than in the non-Zn fertilized plots (~ 29 mg kg^{-1}). Therefore, this small difference may have resulted from Zn being translocated from vegetative tissues to grains during the reproductive stages of wheat (Cakmak 2008). The scant precipitation typical of Mediterranean climate during these stages may have prevented Zn from accumulating in wheat shoots or substantial amounts of Zn from being absorbed from the soil. Because grain Zn contents increased with increasing amount of Zn applied to the soil, we hypothesized that application of increased Zn rates to the soil could enhance Zn uptake. Soil Zn application increased grain Zn concentrations by up to 12.5% here (including both durum, 3 and 6 kg Zn ha^{-1}, and bread wheat, 10 kg Zn ha^{-1}). By contrast, Liu et al. (2019), found application of a 11.7 kg Zn ha^{-1} rate to soil to increase grain Zn concentrations by 36–57% relative to non-Zn fertilized plots. The smaller grain Zn concentrations obtained here may have resulted from the larger $CaCO_3$ content of the Vertisols (> 400 g kg^{-1} as compared to only 45 g kg^{-1}; Liu et al. 2019), which is bound to have reduced soil Zn availability (Alloway 2009).

On the other hand, the foliar treatments increased shoot Zn concentrations before or at the reproductive stages and must thus have enabled Zn re-translocation from these tissues to grains. The foliar Zn treatments that resulted in the higher Zn grain content were those using a rate of 1.28 kg Zn ha^{-1} at early booting (durum wheat) or a later stage (bread what). In fact, these treatments significantly increased Zn grain concentrations (mg kg^{-1}): by 30.5% (38.5 vs. 29.5, 2017–2018 season) when Zn was applied at early booting in durum wheat; 51.0% when Zn was applied at early flowering, and 33.3% when Zn was split between stem elongation (third node detectable) and early flowering (43.5 and 38.1, respectively, vs. 28.8, 2018–2019 season) in bread wheat. These were the only three fertilization strategies that rendered a grain Zn concentrations that fell within the recommended range for biofortified wheat for human nutrition (> 35 mg kg^{-1}; Pfeiffer and McClafferty 2007; Cakmak and Kutman 2018). Similar increases in grain Zn concentration were previously obtained by Ram et al. (2016) by using Zn alone or in combination with pesticides (41.2 and 38.4, respectively, vs. 28.0 mg Zn kg^{-1}) at the booting and milk stages.

The limited effect on grain Zn concentration of the 0.64 kg Zn ha^{-1} rate applied to the aerial part of durum wheat at early booting in relation to the 1.28 kg Zn ha^{-1} rate (11.5 vs. 30.0%, respectively, in the 2017–2018 season) highlights the importance of carefully adjusting the applied rate to the specific crop requirements. Additionally, foliar spraying of Zn at the wrong time (viz., during stem elongation as in one of the treatments in bread wheat, 2018–2019) increased grain Zn concentration inadequately, probably as a result of the foliar area exposed to sprayed Zn—and hence able to absorb it—being too small. These results are consistent with those of Cakmak et al. (2010), who obtained considerably increased grain Zn concentrations by foliar spraying at the booting and milk stages.

Agronomic recommendations for calcareous Vertisols in Mediterranean areas

Although different cultivars were used, wheat grown on Vertisols studied in our field experiments, with low-to-medium available soil P contents (P_{Olsen} = 4–20 mg kg^{-1}), low available soil Zn values (Zn_{DTPA} = 0.16–0.50 mg kg^{-1}) and in an area under Mediterranean climate, exhibited no increase in yield upon Zn fertilization (≤10 kg Zn ha^{-1}, soil applications; 0.64–1.28 kg Zn ha^{-1}, foliar spraying). However, wheat biofortification was observed with appropriate foliar applications of Zn irrespective of wheat yield in the last two field experiments. This was clearly observed in the results obtained in the 2017–2018 season (which was rainy) for durum wheat, and those of the 2018–2019 season (which was dry) for bread wheat. Single Zn applications at rates up to 10 kg ha^{-1} to the soil (relatively low) are not recommended for the target Vertisols as they had little effect on durum and bread wheat yield and biofortification. Also, they resulted in the lowest crop ZnUE values (≤ 1.5% for durum wheat and 0.1% for bread wheat) relative to foliar Zn application (16.2–19.0% in the 2017–2018 season for durum wheat and 3.1–9.4% in the 2018–2019 season for bread wheat) and had no substantial effect on grain or crop Zn uptake. Although different plant species were used in the last two seasons (*T. durum* and *T. aestivum*, respectively) with different sensitivity to Zn deficiency (Cakmak 2008), similar results were obtained regarding yield and biofortification as a function of the application method (soil Zn application and foliar Zn spraying).

Based on grain Zn concentration, ZnUE and Zn crop gain, foliar spraying (1.28 kg Zn ha^{-1}) at the early boot and flowering stages in the 2017–2018 (durum wheat) and 2018–2019 (bread wheat) season, respectively, were the most effective strategies for minimizing the Zn fertilizer rate needed while maximizing wheat biofortification in the short term. Splitting foliar spraying between two different growth stages was seemingly inadvisable for bread wheat (2018–2019) because the resulting increase in grain Zn concentration relative to a single application was too small to justify the cost of two applications. Whenever possible, foliar treatments should be combined with pesticides to avoid unduly raises in costs for farmers. Future experiments should consider applying larger Zn rates to the soil and/or the effect of continuous applications with a view to increasing soil Zn contents and Zn availability, and to comparing their effects with those of foliar treatments. In addition, incorporating the Zn fertilizer into a deeper soil layer—obviously at the expense of greater investments—could be more effective as wheat roots grow deeper and not in the first cm of the soil under Mediterranean climate.

Conclusion

The results of this study on calcareous Vertisols under Mediterranean climate suggests that no yield increase in wheat grown on them is to be expected from Zn fertilization irrespective of application method and rate with single applications at ≤10 kg ha^{-1} to the soil or foliar spraying at 0.64 or 1.28 kg ha^{-1}. Wheat yield was only significantly increased in 1 out of 11 field experiments with Zn application. In the experiment concerned, Zn was applied to the soil at 0.3 or 3 kg Zn ha^{-1} in the 2012–2013 season, where rainfall was relatively high and available Zn relatively low. Foliar Zn applications proved effective for durum and bread wheat biofortification, especially with Zn fertilizer sprayed at 1.28 kg ha^{-1} to plants approaching grain development. These application conditions increased grain Zn concentrations by up to 51.0% as compared to only 12.5% with soil application. Foliar Zn spraying also resulted in the highest Zn use efficiencies and hence minimized Zn fertilizer wastage. Overall, this study reveals that available knowledge on specific Zn fertilization strategies for these Vertisols in Mediterranean areas remains incomplete, so further research into other potential limiting variables (e.g., rainfall, soil P and Zn availability and $CaCO_3$ content) and other scenarios (e.g., Zn fertilization in the long-term with different rotations) is needed.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s11104-021-04863-7.

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Code availability Not applicable.

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Declarations

Ethics approval Not applicable.

Consent to participate Not applicable.

Conflict of interest/competing interests The authors declare no conflict of interest or competing interests.

References

Ahsin M, Hussain S, Rengel Z, Amir M (2020) Zinc status and its requirement by rural adults consuming wheat from control or zinc-treated fields. Environ Geochem Health 42:1877–1892. https://doi.org/10.1007/s10653-019-00463-8

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

Alloway BJ (2008) Zinc in soils and crop nutrition, 2nd edn. IZA and IFA, Brussels, p 139

Brown KH, Rivera JA, Bhutta Z et al (2004) International zinc nutrition consultative group (IZiNCG) technical document #1. Assessment of the risk of zinc deficiency in populations and options for its control. In: food and nutrition bulletin. United Nations University press, Tokyo

Cakmak I (2008) Enrichment of cereal grains with zinc: agronomic or genetic biofortification? Plant Soil 302:1–17. https://doi.org/10.1007/s11104-007-9466-3

Cakmak I, Kalayci M, Kaya Y, Torun AA, Aydin N, Wang Y, Arisoy Z, Erdem H, Yazici A, Gokmen O, Ozturk L, Horst WJ (2010) Biofortification and localization of zinc in wheat grain. J Agric Food Chem 58:9092–9102. https://doi.org/10.1021/jf101197h

Cakmak I, Kutman UB (2018) Agronomic biofortification of cereals with zinc: a review. Eur J Soil Sci 69:172–180. https://doi.org/10.1111/ejss.12437

Cakmak I, Yilmaz A, Kalayci M, Ekiz H, Torun B, Erenoglu B, Braun HJ (1996) Zinc deficiency as a critical problem in wheat production in Central Anatolia. Plant Soil 180:165–172. https://doi.org/10.1007/BF00015299

Catlett KM, Heil DM, Lindsay WL, Ebinger MH (2002) Soil chemical properties controlling $Zinc^{2+}$ activity in 18 Colorado soils. Soil Sci Soc Am J 66:1182–1189. https://doi.org/10.2136/sssaj2002.1182

Chattha MU, Hassan MU, Khan I, Chattha MB, Mahmood A, Chattha MU, Nawaz M, Subhani MN, Kharal M, Khan S (2017) Biofortification of wheat cultivars to combat zinc deficiency. Front Plant Sci 8:1–8. https://doi.org/10.3389/fpls.2017.00281

Chen Y, Barak P (1982) Iron nutrition of plants in calcareous soils. Adv Agron 35:217–240. https://doi.org/10.1016/S0065-2113(08)60326-0

del Campillo MC, Torrent J (1992) Predicting the incidence of Iron Chlorosis in Cal-Careous soils of southern Spain. Commun Soil Sci Plant Anal 23:399–416. https://doi.org/10.1080/00103629209368598

El-Dahshouri MF, El-Fouly MM, Khalifa RKM, El-Ghany HMA (2017) Effect of zinc foliar application at different physiological growth stages on yield and quality of wheat under sandy soil conditions. Agric Eng Int CIGR J Special issue:193–200

Erdal I, Yilmaz A, Taban S, Eker S, Torun B, Cakmak I (2002) Phytic acid and phosphorus concentrations in seeds of wheat cultivars grown with and without zinc fertilization. J Plant Nutr 25:113–127. https://doi.org/10.1081/PLN-100108784

Gee GW, Bauder JW (1986) Particle-size analysis. In: Klute A (ed) Methods of soil analysis, 2nd edn. American Society of Agronomy and Soil Science Society of America, Madison, Madison, Wisconsin, pp 383–412

Graham RD, Welch RM (1997) Trace element intervention strategies in animal and human nutrition a strategy for breeding staple-food crops with high micronutrient density. 447–450

IUSS Working Group WRB (2014) World reference base for soil resources 2014. International soil classification system for naming soils and creating legends for soil maps.

Joy EJM, Stein AJ, Young SD, Ander EL, Watts MJ, Broadley MR (2015) Zinc-enriched fertilisers as a potential public health intervention in Africa. Plant Soil 389:1–24. https://doi.org/10.1007/s11104-015-2430-8

Jurowski K, Szewczyk B, Nowak G, Piekoszewski W (2014) Biological consequences of zinc deficiency in the pathomechanisms of selected diseases. J Biol Inorg Chem 19:1069–1079. https://doi.org/10.1007/s00775-014-1139-0

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

Liu DY, Liu YM, Zhang W, Chen XP, Zou CQ (2019) Zinc uptake, translocation, and remobilization in winter wheat as affected by soil application of Zn fertilizer. Front Plant Sci 10:1–10. https://doi.org/10.3389/fpls.2019.00426

Liu DY, Zhang W, Yan P, Chen XP, Zhang FS, Zou CQ (2017) Soil application of zinc fertilizer could achieve high yield and high grain zinc concentration in maize. Plant Soil 411:47–55. https://doi.org/10.1007/s11104-016-3105-9

Loneragan JF, Webb MJ (1993) Interactions between zinc and other nutrients affecting the growth of plants. In: Zinc in soils and plants. Developments in plant and soil sciences, vol 55. Springer, Dordrecht. https://doi.org/10.1007/978-94-011-0878-2_9

Mabesa RL, Impa SM, Grewal D, Johnson-Beebout SE (2013) Contrasting grain-Zn response of biofortification rice (Oryza sativa L.) breeding lines to foliar Zn application. F Crop Res 149:223–233. https://doi.org/10.1016/j.fcr.2013.05.012

McDonald GK, Genc Y, Graham RD (2008) A simple method to evaluate genetic variation in grain zinc concentration by correcting for differences in grain yield. Plant Soil 306:49–55. https://doi.org/10.1007/s11104-008-9555-y

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

Nriagu J (2019) Zinc deficiency in human health. In: Encyclopedia of environmental health 2th edition. Elsevier, pp 489–499. https://doi.org/10.1016/B978-0-444-52272-6.00674-7

Olsen SR, Cole CV, Watanabe FS, Dean LA (1954) Estimation of available phosphorus in soils by extraction with sodium bicarbonate. USDA Circ 18:45. https://doi.org/10.2307/302397

Pfeiffer WH, McClafferty B (2007) HarvestPlus: breeding crops for better nutrition. Crop Sci 47:S-88–S-105. https://doi.org/10.2135/cropsci2007.09.0020IPBS

Prasad AS (2013) Discovery of human zinc deficiency: its impact on human health and disease. Adv Nutr 4:176–190. https://doi.org/10.3945/an.112.003210

Ram H, Rashid A, Zhang W, Duarte AP, Phattarakul N, Simunji S, Kalayci M, Freitas R, Rerkasem B, Bal RS, Mahmood K, Savasli E, Lungu O, Wang ZH, de Barros VLNP, Malik SS, Arisoy RZ, Guo JX, Sohu VS, Zou CQ, Cakmak I (2016) Biofortification of wheat, rice and common bean by applying foliar zinc fertilizer along with pesticides in seven countries. Plant Soil 403:389–401. https://doi.org/10.1007/s11104-016-2815-3

Ryan J, Ibrikci H, Delgado A et al (2012) Significance of phosphorus for agriculture and the environment in the West Asia and North Africa region. Adv Agron 114:91–143. https://doi.org/10.1016/B978-0-12-394275-3.00004-3

Sacristán D, González-Guzmán A, Barrón V et al (2019) Phosphorus-induced zinc deficiency in wheat pot-grown on noncalcareous and calcareous soils of different properties. Arch Agron Soil Sci 65:208–223. https://doi.org/10.1080/03650340.2018.1492714

Sánchez-Rodríguez AR, del Campillo MC, Torrent J (2017) Phosphorus reduces the zinc concentration in cereals pot-grown on calcareous Vertisols from southern Spain. J Sci Food Agric 97:3427–3432. https://doi.org/10.1002/jsfa.8195

van Wesemael JC (1955) De bepaling van het calciumcarbonaatgehalte van gronden. Chem Weekbl 51:35–36

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 37:29–38. https://doi.org/10.1097/00010694-193401000-00003

Welch RM, Graham RD, Cakmak I (2013) Linking agricultural production practices to improving human nutrition and health. In: expert paper written for ICN2 second international conference on nutrition preparatory technical meeting. Rome, Italy, pp 13–15

Xue Y, Xia H, Christie P, Zhang Z, Li L, Tang C (2016) Crop acquisition of phosphorus, iron and zinc from soil in cereal/legume intercropping systems: a critical review. Ann Bot 117:363–377. https://doi.org/10.1093/aob/mcv182

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

Zasoski RJ, Burau RG (1977) A rapid nitric-perchloric acid digestion method for multi-element tissue analysis. Commun Soil Sci Plant Anal 8:425–436. https://doi.org/10.1080/00103627709366735

Zhang W, Liu D, Li C, Cui Z, Chen X, Russell Y, Zou C (2015) Zinc accumulation and remobilization in winter wheat as affected by phosphorus application. F Crop Res 184:155–161. https://doi.org/10.1016/j.fcr.2015.10.002

Zia MH, Ahmed I, Bailey EH, Lark RM, Young SD, Lowe NM, Joy EJM, Wilson L, Zaman M, Broadley MR (2020) Site-Specific Factors Influence the Field Performance of a Zn-Biofortified Wheat Variety Front Sustain Food Syst:4. https://doi.org/10.3389/fsufs.2020.00135

Zou C, Du Y, Rashid A et al (2019) Simultaneous biofortification of wheat with zinc, iodine, selenium, and iron through foliar treatment of a micronutrient cocktail in six countries. J Agric Food Chem 67:8096–8106. https://doi.org/10.1021/acs.jafc.9b01829

Zou CQ, Zhang YQ, Rashid A, Ram H, Savasli E, Arisoy RZ, Ortiz-Monasterio I, Simunji S, Wang ZH, Sohu V, Hassan M, Kaya Y, onder O, Lungu O, Mujahid MY, Joshi AK, Zelenskiy Y, Zhang FS, Cakmak I (2012) Biofortification of wheat with zinc through zinc fertilization in seven countries. Plant Soil 361:119–130. https://doi.org/10.1007/s11104-012-1369-2