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ORIGINAL PAPER

Biofortification of Forage Peas with Combined Application of Selenium and Zinc Under Mediterranean Conditions

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Abstract

Agronomic biofortification can be used to alleviate the deficient intake of selenium (Se) and zinc (Zn) by livestock. These two essential micronutrients for human and animals play an important role in many physiological functions and biological processes. The aim of the present study was to evaluate the suitability of forage peas, crop with an increasing importance as plant protein source, to be biofortified with a combined treatment of Zn (as $ZnSO_4$-$7H_2O$) and Se (as Na_2SeO_4). A 2-year field experiment was established in southern Spain under semiarid Mediterranean conditions, by following a split-split-plot design. The study year (2017/2018, 2018/2019) was considered the main-plot factor, soil Zn application (50 kg Zn ha^{-1}, nil Zn) as a subplot factor, and foliar application (nil, 10 g Se ha^{-1}, 8 kg Zn ha^{-1}, 10 g Se ha^{-1} + 8 kg Zn ha^{-1}) as a sub-subplot factor. The combined application of 50-kg soil Zn ha^{-1} and the foliar application of 10 g Se ha^{-1} + 8 kg Zn ha^{-1} was the most effective treatment to increase the concentration in forage of Zn and Se, 4-fold and 5-fold, respectively, as well as the Zn bioavailability, forage yield (close to 30%), and crude protein (~ 8%). Thus, forage peas could be considered a very suitable crop to be included in biofortification programs under Mediterranean conditions with Zn and Se as target minerals.

Keywords Sodium selenate · Zinc sulfate · Legumes · Rainfed conditions · Forage yield · Nutritive value parameters

1 Introduction

Forage crops are very important worldwide to provide an outstanding part of the animal feeding in intensive livestock production systems, and as a supplement of the diet in extensive grazing systems. In Spain, around 1.2 million ha are dedicated to forage crops (MAPA 2020). Among them, the use of legume species is highly recommended. This is, among other reasons, because legumes are considered a great protein source in animal feeding, improving, in addition, the physical and chemical soil properties after their cropping. Forage feeding can provide livestock most of its carbohydrate, fat, protein, vitamin, and mineral requirements (Suttle 2010). Mineral content in herbage is clearly linked to their concentration in the soil where plants have been grown and to the capacity of the different plant species to uptake and accumulate them into the edible parts (Fan et al. 2020). Among the essential minerals for animals, most of them are required in so tiny amounts or are so abundant into soil that plants fulfill their requirements easily. However, some minerals such as selenium (Se) or zinc (Zn), frequently appear in scarce concentrations in soil, providing forage with inadequate concentrations of them and causing, consequently, a variable degree of Se and/or Zn deficiency to livestock.

Selenium concentration in many soils all over the world, although very variable, can be considered low, ranging between 10 and 2000 μg kg^{-1}, with an average value of 400 μg kg^{-1} (Arthur 2003). In the southwestern Iberian Peninsula, several studies have established values of Se in soils of about 140 μg total Se kg^{-1} and 12 μg extractable Se kg^{-1} (Poblaciones et al. 2014; Rodrigo et al. 2014). These levels can be considered deficient to marginal according to the classification given by Dinh et al. (2018). In relation to Zn, its concentration in soils worldwide ranges between 10

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and 300 mg kg^{-1} (Alloway 2009). Studies of Gomez-Coronado et al. (2016) in the southwest of the Iberian Peninsula showed values lower than 25 mg total Zn kg^{-1} and 0.3 mg Zn-DTPA kg^{-1}, considered also very deficient according to Alloway (2009).

Selenium plays in livestock an essential role in many physiological functions and biological processes (Ghaderzadeh et al. 2016), integrating many mammalian enzymes, such as glutathione peroxidases (Reich and Hondal 2016) and selenoproteins (Allmang and Krol 2006). The recommended values of Se intake for livestock range from 0.1 to 0.5 mg Se kg^{-1} feed dry matter (DM; Suttle 2010). A deficient intake of Se can cause in cattle and sheep a large number of diseases, such as reduced growth and white muscle disease (nutritional muscular dystrophy), a myopathy of heart and skeletal muscle in young animals, or poor reproductive performance in older animals (Mehdi and Dufrasne 2016). In swine, Se deficiency can cause hepatosis dietetica, mulberry heart disease, or pancreatic fibrosis (Oropeza-Moe et al. 2015). Regarding Zn, it is involved in enzyme systems and protein synthesis, but also in carbohydrate metabolism and other biochemical processes. The required amount of Zn by livestock is about 35 mg Zn kg^{-1} feed DM (Suttle 2010), although it might depend on the livestock type. Its deficiency can causes numerous disorders in livestock, such as skin parakeratosis, reduced or cessation of growth, general debility, lethargy, and increased susceptibility to infection (Hill and Shannon 2019).

In those conditions of low soil concentrations, in order to prevent or limit Se and Zn deficiency, their intake should be highly increased to reach the recommended values. Among the different strategies to achieve this increase, the agronomic biofortification of crops has demonstrated to be very successful in a wide range of plants (White and Broadley 2005). This practice has been intensively studied in crops for human food, such as wheat, rice, or chickpea (Germ et al. 2013; Ram et al. 2016; Hla Hla et al. 2019; Manojlović et al. 2019), and to a lesser extent in grain crops for animal feeding (Rodrigo et al. 2013; Novoselec et al. 2018). The very few studies which have been conducted in forage crops deal mainly with alfalfa (Petković et al. 2019). Among forage crops, legume species seems to be especially suitable for agronomic biofortification, since many studies have demonstrated a higher capacity to accumulate Se and Zn in their tissues (Poblaciones et al. 2014; Poblaciones and Rengel 2016). Although several legumes can be used with this purpose, forage peas have a high productive potential in the semiarid Mediterranean climate of Spain under rainfed conditions because of their drought and diseases tolerance, and excellent nutritional value. Also, this crop is included in the greening payment of European CAP subsidies. As a consequence of all of these aspects, peas are the most cultivated legume in Spain with more than 173,000 ha (MAPA 2020), although mainly as field peas.

Biofortification of field or market peas has already been demonstrated to be successful with either Se (Gawalko et al. 2009; Thavarajah et al. 2010; Poblaciones et al. 2013; Poblaciones and Rengel 2018) or Zn (Poblaciones and Rengel 2016), but very few is known about its suitability in forage peas. In those studies, the optimal application conditions were established. For Se, there is a broad agreement to consider a foliar application of 10 g Se ha^{-1} at the start of the flowering stage the most effective. In many studies, the use of sodium selenate seems to be the most appropriated, although potassium selenate might be also more efficient than other less-soluble forms as barium selenate or sodium selenite (Broadley et al. 2006; Poblaciones et al. 2013). For Zn, although this general consensus lacks, many studies may have suggested the application of zinc sulfate ($ZnSO_4$-$7H_2O$) as either a soil application before sowing (50 kg Zn ha^{-1}) or a foliar application at the start of flowering (4–8 kg Zn ha^{-1}), each one alone or in combination. There are other forms of Zn used, like ZnEDTA, which might be more efficient but its higher cost made it less accessible to farmers (Cakmak 2008; Das et al. 2019).

When soils are deficient in several micronutrients, a simultaneous biofortification might be more effective and profitable for farmers, as previously suggested by Zou et al. (2019) in other crops, such as wheat. In peas, the combined Se and Zn biofortification has demonstrated to be effective in the increase of Se and Zn in the grain under greenhouse conditions (Poblaciones and Rengel 2017). In addition to the total mineral concentration in forage, the bioavailability of these minerals and the presence of certain anti-nutritional components, mainly phytic acids (myo-inositol 1,2,3,4,5,6-hexakisphosphate), are also important to be evaluated, due to their known capacity in reducing mineral bioavailability and absorption by animals (Gupta et al. 2015). Thus, according to the previous literature indicated above, the use of forage peas in biofortification programs applying simultaneously Zn and Se seems to have a great potential in animal feeding, but it needs to be evaluated on the real semiarid Mediterranean conditions in the field and using forage as the main harvesting product instead of grain.

Therefore, in order to contribute to nutrient deficiency alleviation in livestock, the general aim of the present study was to evaluate the suitability of forage peas to be biofortified by Zn and Se in field conditions under a semiarid Mediterranean climate. With the aim of analyzing the best application strategy to boost the Se and Zn accumulation in forage, their bioavailability (via phytate concentration determination), and its effect on productive (forage yield) and nutritive value parameters (protein, fiber, lignin, ashes, digestibility, and quality indexes and concentration of several nutrients, like magnesium [Mg], calcium [Ca], and iron [Fe]), foliar Se and foliar Zn were applied alone or in combination with soil Zn application to a forage peas crop during two consecutive growing seasons.

2 Material and Methods

2.1 Site, Experimental Design, and Crop Management

A field experiment was conducted in Badajoz, southern Spain (38° 54′ N, 6° 44′ W, 186 m above sea level), in a Xerofluvent soil (USDA 1998) under rainfed Mediterranean conditions in the 2017/2018 and 2018/2019 growing seasons. Weather-related parameters for this area for the study years, as well as for the average year obtained from a 30-year period, are shown in Fig. 1. All climate data were taken from a weather station located at the study site. As expected, because of the great irregularity characteristic of the Mediterranean climate, the two study years were substantially different. In 2017/2018, rainfall was quite similar to that of the average, although March and April were two very rainy months, accounting almost 252 mm, precipitation values much higher than on average. The second study year (2018/2019), however, can be considered a very dry year, since total rainfall (295 mm in total) was close to 35% lower than on average, with important drought periods during February and March.

The experiment was arranged as a split-split-plot design with four replicates randomly distributed, including the year (2017/2018 and 2018/2019) as the main-plot factor, Zn soil application (2 treatments, i: without any application [0SZn] and ii: a soil application of 50 kg $ZnSO_4$-$7H_2O$ ha^{-1} [50SZn]) as the subplot factor, and foliar application (4 treatments, i: without any application [0F], ii: two foliar applications of 4 kg $ZnSO_4$-$7H_2O$ ha^{-1} each at the start of flowering and 2 weeks later [8FZn], iii: a foliar application of 10 g Se ha^{-1} as Na_2SeO_4 at the start of flowering [10FSe], and iv: a combination of ii and iii [8FZn+10FSe]) at the start of flowering as the sub-subplot factor.

Plot size for each treatment was 15 m^2 (3 m × 5 m). Before the sowing of the first season (in October 2017), Zn soil treatment was carried out with 50 kg $ZnSO_4$-$7H_2O$ ha^{-1} which was sprayed to the soil surface and then incorporated into the soil by conventional tillage. Soil treatment was only made at the beginning of the first year of the experiment, in order to evaluate its residual effect along the essay duration. For the foliar Zn treatment, 4 kg $ZnSO_4$-$7H_2O$ ha^{-1} was diluted in 800 L H_2O ha^{-1} to obtain a 0.5% (w/v) solution. This volume was selected as it allowed a very uniform and regular application. The treatment was applied with a backpack sprayer at the start of the flowering stage late in the day, to avoid an eventual burning in plants. With this procedure, which might resemble a real application in field, mainly the adaxial side of the leaf was sprayed. Foliar treatment was repeated 2 weeks later. Finally for the Se treatment, 10 g Na_2SeO_4 ha^{-1} was diluted in 800 L H_2O ha^{-1} to obtain a 0.003% (w/v) solution and applied as in the case of foliar Zn. Due to the high solubility of the fertilizers, the use of any adjuvant was not necessary. These treatments, volumes, sprayers, etc. were chosen based on the previous experience of Cakmak et al. (2010), Poblaciones et al. (2013), and Gomez-Coronado et al. (2016) in similar conditions. All the treatments were arranged at the same position in the two experimental years. Residual effect of foliar applications could be ruled out due to the low dose rates used for the treatments.

The forage pea (*Pisum sativum* L.) cultivar used was "Guifredo." Conventional tillage treatment was used to prepare a proper seedbed before sowing. The sowing was performed at a rate of 160-kg seeds ha^{-1}, in rows of 20 cm in early November in the first year (2017) and late December in the second year (2018). Sowing delay in the second year was due to the intense precipitations in November which did not allow an earlier sowing. No further fertilization, other than that of Zn and Se, was applied since soil phosphorus (P) and potassium (K) levels were adequate. Weed control was carried

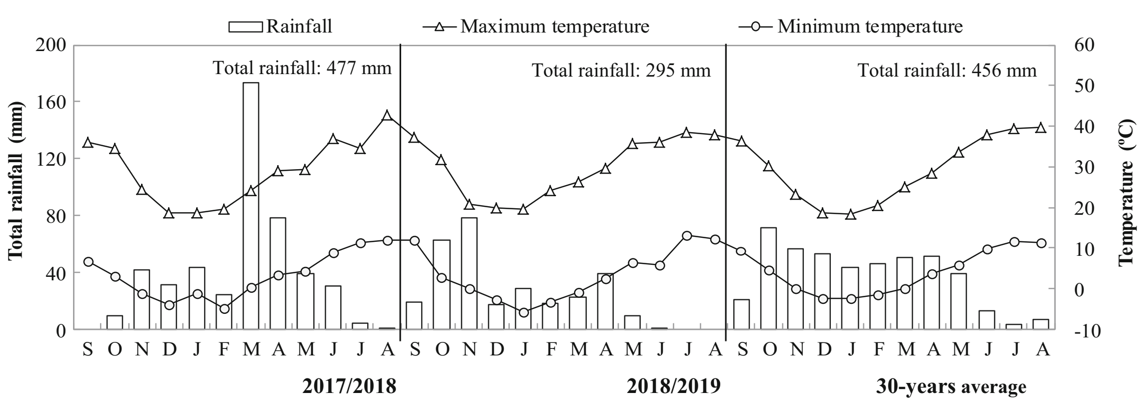

Fig. 1 Monthly and annual rainfall and mean maximum and minimum temperatures in 2017/2018, 2018/2019, and an average year from a 30-year period in Badajoz (Spain)

out by applying Afalon 50 WP (wettable powder containing 500 g kg^{-1} linuron; AAKO, Leusden, The Netherlands) in the sowing day. Forage harvest took place in late April and May, in 2018 and 2019 respectively.

2.2 Soil Analysis

Before any treatment, four representative soil samples of 30 cm deep were taken from the experimental site. Soil samples were air dried and sieved to < 2 mm using a roller mill. Texture was determined granulometrically (Day 1965), soil pH was measured using a calibrated pH meter (ratio, 10-g soil:25-ml deionized H_2O), and soil organic matter (SOM) was determined by oxidation by dichromate (Walkley and Black 1934). Electrical conductivity (EC) was determined using an EC meter. Total N was determined using the Kjeldahl method (Bremner 1996), by means of a Kjeltec™ K350 distillation Unit (Buchi Ltd., Flawil, Switzerland). Extractable P was determined by using the Olsen procedure, and K and sodium (Na) were extracted with ammonium acetate (1 N) and quantified by atomic absorption spectrophotometry (Helyos alpha, 9423-UVA, Unicam, Cambridge, UK).

Extractable Ca, Mg, Fe, and Zn were determined by using DTPA (diethylenetriaminepentaacetic acid) at a soil:solution ratio of 1:2 and shaking time of 2 h at 120 rpm. Concentrations of those minerals were determined using an inductively coupled plasma mass spectrometer (ICP-MS) (Agilent 7500ce, Agilent Technologies, Palo Alto, CA, USA) operating in the hydrogen gas mode—this analytical method was developed by the Elemental and Molecular Analysis Service of the University of Extremadura (Spain). To evaluate the residual effect in those Zn treatments with a soil application, four additional samplings (with their corresponding extractable Zn determination) were performed along the experiment: in January and in harvest time, in both growing seasons. Finally, extractable Se was determined by using KH_2PO_4 (0.016 mM, pH 4.8) at a ratio of 10-g dry weight soil:30 ml KH_2PO_4 w/v (Zhao and McGrath 1994). The Se concentration in the extracts was determined by ICP-MS, as described above. For quality assurance in each batch of samples, blanks and an internal patron were used as reference material (1.12 mg Zn-DTPA kg^{-1} and 10 μg extractable Se kg^{-1}), being the recovery of 95 and 90%, respectively. All the results were reported on a dry weight basis.

2.3 Forage Analyses

Once harvested, forage was oven dried at 70 °C until constant weight and then the DM recorded. The dried samples, after grinding, were also used to determine the main quality parameters. Thus, total N content was analyzed by using the Kjeldahl method (Kjeltec™ 8200 Auto Distillation Unit. FOSS Analytical. Hillerod, Denmark) and used to estimate crude protein (CP) by multiplying the biomass N × 6.25 (Sosulski and Imafidon 1990). Official procedures (AOCS 2006) were followed to determine neutral detergent fiber (NDF), acid detergent fiber (ADF), and acid detergent lignin (ADL) by means of a fiber analyzer (ANKOM8–98, ANKOM Technology, Macedon, NY). Total ash content was determined by ignition of the sample in a muffle furnace at 600 °C, such as that indicated in the official procedure (AOCS 2006). The relative feed value (RFV) of dry matter and the organic matter digestibility (OMD) of the forage were calculated by following the procedure proposed by Linn and Martin (1991) and Chibani et al. (2010), respectively.

Forage Zn, Se, Ca, Fe, and Mg concentrations were also determined as follows: forage of each treatment was finely grounded (< 0.45 mm) using an agate ball mill (Retch PM 400 mill); a 1-g aliquot was digested with ultra-pure concentrated nitric acid (2 ml) and 30% w/v hydrogen peroxide (2 ml) using a closed-vessel microwave digestion protocol (Mars X, CEM Corp, Matthews, NC) and diluted to 25 ml with ultra-purified water (Adams et al. 2002). Sample vessels were thoroughly washed with acid before use. For quality assurance, a blank and a standard reference material (tomato leaf, NIST 1573a) were included in each batch of samples. The nutrient specific recovery was 94% compared with certified reference material values. Concentrations of Zn, Se, Ca, Fe, and Mg were determined by ICP-MS as described above for soil samples. In order to consider the dilution effect caused by the different forage yield between growing seasons, total content of each mineral per ha was also determined by multiplying its concentration in forage and forage yield.

Forage Zn bioavailability was obtained by determining the phytate concentration, estimated through phytic acid. Phytic acid was determined by following the procedure described by Thavarajah et al. (2009), based on precipitation of ferric phytate and measurement of the supernatant remaining iron (Fe). Phytic acid was extracted from about 0.2 g of ground field pea biomass in 10 mL of 0.2 M HCl (pH 0.3) after shaking for 2 h. One milliliter of supernatant was treated with 2 mL of ferric solution ($NH_4Fe(SO_4)_2$-$12H_2O$) in a boiling water bath for 30 min. After cooling, samples were centrifuged, and 1 mL of supernatant was treated with 1.5 mL of 0.064 M bipyridine (2-pyridin-2-ylpyridine, $C_{10}H_8N_2$) to measure Fe. After mixing, the solution was incubated for 10 min at room temperature, and absorbance was measured with a spectrophotometer at 419 nm. Relation between phytates and Zn, Se, Ca, Fe, or Mg molar ratio was calculated.

2.4 Statistical Analysis

The effect of the Zn soil application on the concentration of extractable Zn in soil was evaluated by a mixed-design analysis of variance model (or split-plot ANOVA), including the main-plot factor "sampling time" (before starting, in January and harvest of the 1st growing season, and in January and harvest of the 2nd growing season), the subplot factor "Zn application" (0SZn+0F, 50SZn+0F, and 50SZn+8FZn), and its interaction in the model, in order to analyze its residual effect. Data of Se and Zn concentration in forage, its phytate concentration (estimated through phytic acid), and the phytate/mineral molar ratios, as well as forage yield, nutritive value parameters (CP, NDF, ADF, ADL, RFV, and DMO), and the mineral concentration in forage (Ca, Fe and Mg), were subjected also to mixed-design models, in this case to split-split-plot ANOVAs, including the main-plot factor "year" (2017/2018 and 2018/2019), the subplot factor "soil Zn application" (0SZn and 50SZn), the sub-subplot factor "foliar application" (0F, 8FZn, 10FSe, and 8FZn+10FSe), and their interactions in the model. When significant differences were found in ANOVA, means were compared using Fisher's protected least significant difference (LSD) test at $P \leq 0.05$. In order to normalize the variable distribution as well as to stabilize the variance of residues, the transformation $\sqrt{x}$ was performed for forage yield and $\mathrm{Ln}(x + 1)$ for the concentration of Zn into soil and Fe and Se in forage. All these analyses were performed with the Statistix v. 8.10 package.

3 Results

3.1 Soil Characteristics and Extractable Zn Evolution into Soil During the Experiment

The analysis of the soil samples of the study site indicated a clay loamy soil with slightly acid pH (6.4 ± 0.02; mean ± standard error), without problems of salinity (electrical conductivity = 1321.4 ± 24.04 μS cm^{-1}) and with a very low organic matter content (SOM) (1.31 ± 0.09%). This information, as well as the soil mineral concentrations, can be observed in Table 1. Among them, the extractable Se concentration in the topsoil could be considered very low with a value of 1.27 ± 0.01 μg Se kg^{-1}, and the Zn-DTPA low, with a value of 0.38 ± 0.08 mg kg^{-1}. The split-plot ANOVA performed to evaluate the residual effect of the Zn applications in the concentration of Zn into the topsoil showed sampling time (degree of freedom, df = 4, F value = 5.24, $P = 0.0226$), Zn application (df = 2, F value = 44.99, $P < 0.001$), and their interaction (df = 8, F value = 3.26, $P = 0.0152$) to be all significant variables (considering a $P \leq 0.05$). The Zn concentration into topsoil increased after the soil application in October 2017, and then, no significant variation was found during the 2 years of the experiment, regardless of the foliar application, accounting an average value of 1.22 mg kg^{-1} of extractable Zn-DTPA after the application (Fig. 2).

3.2 Zn and Se Concentrations and Contents in Forage and Its Bioavailability

Zn concentration and total Zn content were both affected by the main effects "study year" and "foliar application" and by their interaction (Table 2). For both response variables, only the foliar treatments containing Zn provided forage with a higher Zn concentration in comparison with the controls (Fig. 3 and Fig. S1). The Se application did not affect the Zn accumulation in forage. The study year modulated the intensity of the effect of the foliar Zn application on the Zn concentration, reaching the highest accumulation values in 2017/2018 (Fig. 3). When the total Zn content was considered to avoid an eventual dilution effect, as the study year affected forage yield, the highest values were also reached in 2017/2018 (Fig. S1).

As expected, the foliar treatments containing Se were significantly the most effective in boosting the Se concentration in forage (Table 2 and Fig. 4). A very interesting synergic effect was observed between Se and Zn, as the foliar treatments including both minerals produced the highest Se accumulation in the forage in comparison with the corresponding

Table 1 Soil properties expressed as mean ± standard deviation (SE) from four samples ($n = 4$). An interpretation of such values for cropping is also shown

Parameter	Value	Interpretation*
Texture		Clay loam
pH (H_2O)	6.4 ± 0.02	Slightly acidic
Electrical conductivity (μS cm^{-1})	1321.4 ± 24.04	Very slightly saline
Organic matter (%)	1.31 ± 0.09	Very low
Total N (%)	0.12 ± 0.007	Medium
P Olsen (g P kg^{-1})	4.9 ± 0.05	Low
Assimilable K (meq K 100 g^{-1})	0.82 ± 0.02	Low
Exchangeable Ca (meq Ca 100 g^{-1})	6.23 ± 0.67	Low
Exchangeable Na (cmol Na kg^{-1})	0.26 ± 0.01	Very low
Extractable Se (μg Se kg^{-1})	1.27 ± 0.01	Very low
Zn-DTPA (mg Zn kg^{-1})	0.38 ± 0.08	Low
Exchangeable Mg (meq Mg 100 g^{-1})	3.72 ± 0.37	Medium

*According to Hazelton and Murphy (2016)

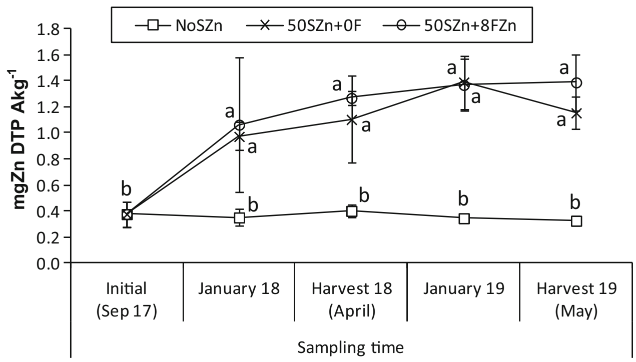

Fig. 2 Zn-DTPA concentration into topsoil of the study area as affected by the interaction "sampling time (5 times)*Zn application (3 treatments: NoSZn, 50SZn+0F and 50SZn+8FZn)." Error bars indicate standard error ($n = 3$). Different letters mean significant differences between means according to the LSD test ($P \leq 0.05$). Although the LSD test was performed on the transformed variable, back-transformed values are presented to ease interpretation

treatment without Zn, i.e., when foliar Se was applied, the Se accumulation was significantly higher if foliar Zn was also applied, but when Se was not applied, the mere foliar Zn application also caused an increase of the Se concentration in forage (Fig. 4). When the total Se content per ha was considered instead of Se concentration to avoid an eventual dilution effect, the interaction "study year*foliar application" also showed a significant influence (Table 2). In this case, the synergic effect was also evidenced, but especially in 2017/2018, and only when Se was also applied (Fig. S2).

Phytic acid in forage was only affected by the interaction "study year*Zn soil application." However, the molar ratios between phytates and both Zn and Se were mainly influenced by the "foliar application," although the effect of this factor was mostly dependent on the study year (Table 2). Soil Zn application increased the concentration of phytic acid, but only in 2017/2018 (Table 3). Foliar Zn application clearly decreased phytate:Zn ratio, as expected considering that these treatments had broadly increased the Zn concentration in forage. This effect, even when present in both study years, was more defined in 2017/2018 (Table 3). Also as expected, foliar Se application clearly decreased phytate:Se ratio, but the foliar Zn application alone also caused a diminution in this parameter, although of a lower intensity (Table 3).

3.3 Effect of Zn and Se Application on Forage Yield and Nutritive Value Parameters

The analysis of the forage showed that the "study year" clearly and significantly affected almost all the parameters studied as main factor, but also when its interaction with "Zn soil

Table 2 Summary of the split-split-plot ANOVAs ($n = 4$) showing the effect of the main-plot factor (year), subplot factor (Zn soil application), sub-subplot factor (foliar application), and their interactions on each forage parameter evaluated. DF, degree of freedom; F values, including the level of significance (*$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$), are shown in the rest of the rows

	Year (Y)	Zn soil applic. (S)	Foliar applic. (F)	Y*S	Y*F	S*F	Y*S*F
DF	1	1	3	1	3	3	3
Zn	26.91*	0.31	135.18***	0.75	9.84***	0.11	1.08
Se	133.4**	0.01	79.10***	0.26	0.32	0.52	0.08
TZn	78.18**	5.59	62.58***	0.10	7.70***	0.37	0.62
TSe	277.6***	2.30	31.28***	0.04	7.60***	0	0.19
Phytic acid	1.24	2.87	0.80	11.19*	0.76	1.01	0.63
Ph/Zn	0.54	2.19	198.0***	0.15	8.88***	1.02	0.47
Ph/Se	122.27**	1.04	61.16***	0.01	5.25**	0.51	0.04
Yield	10.67*	14.72**	0.41	0.00	0.01	0.08	0.04
CP	479.3***	0.90	5.06**	5.15	1.59	0.03	1.14
NDF	124.6**	0.02	0.84	0.87	2.17	0.72	0.23
ADF	882.7***	0.44	0.67	2.24	0.56	0.94	0.87
ADL	982.5***	10.21*	0.49	4.74	0.65	0.34	0.18
Ashes	190.5***	8.85*	3.30*	6.85*	1.57	0.90	1.49
RFV	2343.6***	2.82	0.25	0.04	1.38	0.13	0.59
OMD	8855.2***	2.57	1.21	0.60	0.86	0.17	0.23
Mg	64.27**	1.30	6.62**	8.1*	8.62***	0.57	0.90
Ca	6.21	7.11*	3.79*	19.58**	7.76***	0.31	0.53
Fe	160.9**	0.15	6.25**	2.56	2.16	1.19	0.07
Ph/Mg	85.7**	2.58	9.08***	6.98*	14.54***	1.03	0.35
Ph/Ca	2.12	3.60	4.30*	2.36	5.71**	0.21	0.65
Ph/Fe	146.9**	0.74	4.11*	6.05*	0.80	1.78	0.02

TZn, total Zn content = Zn*yield; *TSe*, total Se content = Se*yield; *Ph/mineral*, molar ratio phytate/each mineral; *Yield*, forage yield; *CP*, crude protein; *NDF*, neutral detergent fiber; *ADF*, acid detergent fiber; *ADL*, acid detergent lignin; *RFV*, relative forage value; *OMD*, organic matter digestibility

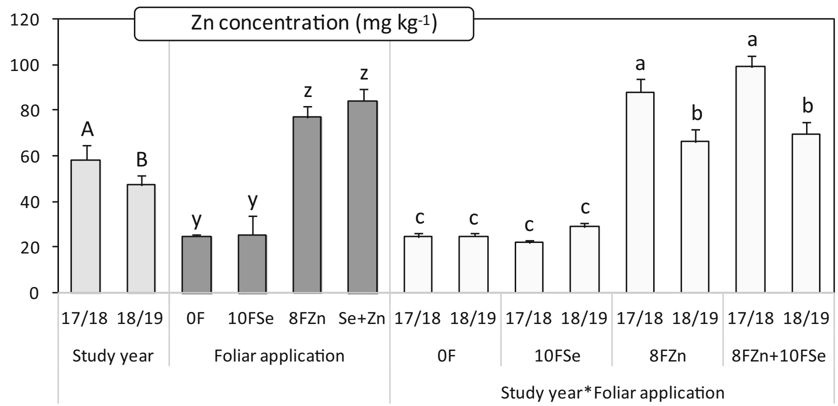

Fig. 3 Concentration of Zn in the forage as affected by the main effects "study year (Y)," "foliar application (F)," and by their interaction (Y*F). Charts indicate means ($n = 4$), and error bars indicate standard error. Within each factor, different letters mean significant differences between means according to the LSD test ($P \leq 0.05$). In order to make the differences clearer, a different set of letters was assigned to each factor (lowercase letters [a, b, c, d] for "Y*F," lowercase letters [z, y] for "F," and uppercase letters [A, B] for "Y")

application" and "foliar application" was analyzed (Table 2). Considering the "study year" the main effect, in 2017/2018, the forage yield, CP, fibers (NDF and ADF) and lignin (ADL), ashes, the concentration of Fe, and the molar ratio phytate:Mg showed higher values than in 2018/2019, while the RFV (145.3 ± 2.4 in 2018/2019 vs 103.8 ± 1.8 in 2017/2018), OMD (58.6 ± 0.2% in 2018/2019 vs 52.8 ± 0.2% in 2017/2018), Mg concentration, and the molar ratio phytate:Fe were higher in 2018/2019 (Tables 3 and 4, and Fig. 5).

Besides "study year", forage yield was significantly affected by the main effect "Zn soil application" (Table 2). When 50 kg $ZnSO_4$-$7H_2O$ ha^{-1} was applied to the soil, forage yield increased nearly 30%, from around 6933 on average in the no-soil Zn application situation to around 8944 kg DM ha^{-1} on average after Zn application (Fig. 5a). In relation to the nutritive value parameters of the forage, soil Zn application also

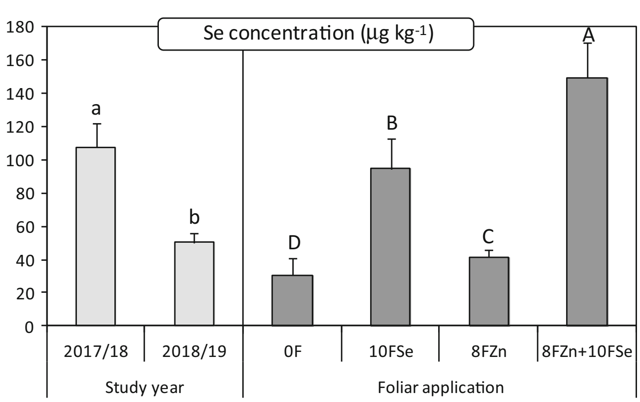

Fig. 4 Concentration of Se in the forage as affected by the main effects "study year (Y)" and "foliar application (F)." Charts indicate means ($n = 4$), and error bars indicate standard error. Within each factor, different letters mean significant differences between means according to the LSD test ($P \leq 0.05$). In order to make the differences clearer, a different set of letters was assigned to each factor (lowercase letters for "Y" and uppercase letters for "F"). Although the LSD test was performed on the transformed variable, back-transformed values are presented to ease interpretation

significantly affected ADL and ashes (Table 2). When soil Zn was applied, lignin content in forage was lower comparing with no-soil Zn application (6.59% vs 7.08% on average, respectively, Fig. 5d), while the ash content significantly increased ($P < 0.05$) under the soil Zn application (50SZn treatment: ashes = 0.29 ± 0.04%, 0SZn treatment: ashes = 0.25 ± 0.03%). Ashes were also affected by the main effect "foliar application" (Table 2), producing significantly the lowest values when both foliar Zn and Se (treatment 8FZn+10FSe) were applied, with a value of 0.20 ± 0.04%. The treatments containing foliar Zn produced significantly the highest values of CP, regardless of whether foliar Se was also applied or not. When foliar Se was applied alone, CP in the forage was not significantly different from that of the control forage, without foliar application (Fig. 5b). Finally, RFV and OMD were not affected by any application either soil or foliar.

In relation to the mineral accumulation in forage, all the minerals analyzed (Mg, Ca, and Fe) were affected by either "foliar" or "soil application" alone or in interaction with the "study year" (Table 2). Thus, when "foliar application" was analyzed as the main effect, Mg concentration increased with the Se application in relation to control, but only when it was applied alone, not when foliar Zn was also applied. However, when each year was analyzed separately, such an effect was not so clearly observed (Table 4); thereby, soil Zn application reduced the Mg concentration in 2017/2018. The concentration of Ca in forage decreased when soil Zn was applied, especially in 2017/2018. Foliar Se seemed to increase the accumulation of Ca when it was applied alone, but such an effect is unclear when each year was evaluated separately (Table 4). Finally, the Fe concentration in forage tended to decrease when foliar Zn was applied (Table 4).

When the bioavailability of Mg, Ca, and Fe was analyzed through the molar ratios phytates:mineral, soil Zn application increased the ratio phytate:Mg in forage, but only in 2017/2018 (Table 3). Foliar Zn application also increased the ratio

Table 3 Phytate concentration (estimated through phytic acid) in the forage and the molar ratio phytate:each mineral (Zn, Se, Mg, Ca, and Fe), expressed as mean value ± standard error ($n = 4$) as affected by the main effects "study year (Y)," "Zn soil application (S)," and "foliar application (F)" (in italics) and by the interactions "Y*S" and/or "Y*F". Within each parameter and factor, different letters mean significant differences between means according to the LSD test ($P \leq 0.05$). In order to make the differences clearer, a different set of letters was assigned to each factor (lowercase letters [a, b, c, d] for "Y*F", lowercase letters [z, y, x] for "Y*S", Greek letters for "S", uppercase letters [Z, Y] for "F", and uppercase letters [A, B] for "Y"). If letters do not appear, this factor did not have a significant effect according to split-split-plot ANOVA

Mineral	Factor	Treatment	Study year		
			2017/2018	2018/2019	Average
Phytic acid (g kg^{-1})	Zn soil application	0SZn	6.89 ± 0.02 y	6.92 ± 0.02 zy	*6.91 ± 0.02*
		50SZn	6.97 ± 0.02 z	6.90 ± 0.02 y	*6.93 ± 0.02*
	Average		*6.93 ± 0.02*	*6.91 ± 0.01*	
Phytate:Zn	Foliar application	0F	29.1 ± 1.9 ab	28.1 ± 1.3 b	*28.6 ± 1.1 Z*
		10FSe	31.7 ± 1.3 a	24.6 ± 1.6 c	*28.1 ± 1.3 Z*
		8FZn	8.0 ± 0.6 de	10.9 ± 1.0 d	*9.4 ± 0.7 Y*
		8FZn+10FSe	7.0 ± 0.4 e	10.1 ± 0.7 de	*8.5 ± 0.6 Y*
	Average		*19.0 ± 2.1*	*18.4 ± 1.6*	
Phytate:Se	Foliar application	0F	22.6 ± 2.7 c	41.6 ± 3.1 a	*32.1 ± 3.6 Z*
		10FSe	6.9 ± 0.4 f	13.2 ± 1.2 de	*10.0 ± 1.0 X*
		8FZn	16.1 ± 1.7 d	31.1 ± 2.6 b	*23.6 ± 2.4 Y*
		8FZn+10FSe	4.9 ± 1.0 f	9.7 ± 0.6 ef	*7.3 ± 2.7 X*
	Average		*19.0 ± 1.5 B*	*23.9 ± 2.6 A*	
Phytate:Mg	Zn soil application	0SZn	0.13 ± 0.01 y	0.11 ± 0.00 x	*0.12 ± 0.00*
		50SZn	0.15 ± 0.01 z	0.10 ± 0.00 x	*0.13 ± 0.01*
	Foliar application	0F	0.12 ± 0.00 c	0.11 ± 0.01 cd	*0.12 ± 0.00 Y*
		10FSe	0.12 ± 0.01 c	0.10 ± 0.01 d	*0.11 ± 0.01 Y*
		8FZn	0.15 ± 0.01 b	0.10 ± 0.00 d	*0.13 ± 0.01 Z*
		8FZn+10FSe	0.17 ± 0.01 a	0.10 ± 0.00 d	*0.13 ± 0.01 Z*
	Average		*0.14 ± 0.00 A*	*0.10 ± 0.00 B*	
Phytate:Ca	Foliar application	0F	0.05 ± 0.00 bc	0.04 ± 0.01 c	*0.05 ± 0.00 YX*
		10FSe	0.04 ± 0.01 c	0.05 ± 0.00 bc	*0.04 ± 0.00 X*
		8FZn	0.06 ± 0.00 ab	0.05 ± 0.00 c	*0.05 ± 0.00 ZY*
		8FZn+10FSe	0.06 ± 0.00 a	0.04 ± 0.00 c	*0.05 ± 0.00 Z*
	Average		*0.05 ± 0.00*	*0.04 ± 0.00*	
Phytate:Fe	Zn soil application	0SZn	0.34 ± 0.05 x	0.88 ± 0.04 z	*0.61 ± 0.05*
		50SZn	0.42 ± 0.08 x	0.71 ± 0.04 y	*0.57 ± 0.04*
	Foliar application	0F	0.32 ± 0.06	0.79 ± 0.04	*0.56 ± 0.07 YX*
		10FSe	0.28 ± 0.03	0.72 ± 0.06	*0.50 ± 0.07 Y*
		8FZn	0.39 ± 0.03	0.85 ± 0.07	*0.62 ± 0.07 ZY*
		8FZn+10FSe	0.52 ± 0.10	0.84 ± 0.05	*0.68 ± 0.07 Z*
	Average		*0.38 ± 0.03 B*	*0.80 ± 0.03 A*	

In the case of Fe concentration, although the LSD test was performed on the transformed variable, back-transformed values are presented to ease interpretation

phytate:Mg in 2017/2018, especially when foliar Se was also applied. A similar foliar application effect happened in relation to the ratio phytate:Ca. Here, also the foliar Zn application increased such values but again only in 2017/2018 (Table 3). Phytate:Fe molar ratio was decreased by the soil Zn application in the second study year (2018/2019), but this ratio increased with foliar Zn application, when "foliar treatment" was considered the main effect.

4 Discussion

The analysis of the soil conditions of the study site indicated a low or a very low availability of Zn and Se, respectively. The soil concentration of these minerals is the major factor affecting their accumulation in the edible parts of plants. This was supported by the fact that in the non-fertilized plots, the concentration of total Zn and Se in the forage of peas was on

Table 4 Concentration of Mg, Ca, and Fe in the forage (expressed as mean value ± standard error; $n = 4$) as affected by the main effects "study year (Y)", "Zn soil application (S)", and "foliar application (F)" (in italics) and by the interactions "Y*S" and "Y*F". Within each parameter and factor, different letters mean significant differences between means according to the LSD test ($P \leq 0.05$). In order to make the differences clearer, a different set of letters was assigned to each factor (lowercase letters [a, b, c, d] for "Y*F", lowercase letters [z, y, x] for "Y*S", Greek letters for "S", uppercase letters [Z, Y] for "F", and uppercase letters [A, B] for "Y"). If letters do not appear, this factor did not have a significant effect according to split-split-plot ANOVA

Mineral	Factor	Treatment	Study year		
			2017/2018	2018/2019	Average
Mg (g kg^{-1})	Zn soil application	0SZn	1.99 ± 0.08 y	2.40 ± 0.07 z	*2.20 ± 0.07*
		50SZn	1.71 ± 0.11 x	2.53 ± 0.06 z	*2.11 ± 0.08*
	Foliar application	0F	2.03 ± 0.06 d	2.30 ± 0.10 bc	*2.16 ± 0.07 Y*
		10FSe	2.11 ± 0.11 cd	2.59 ± 0.13 a	*2.35 ± 0.10 Z*
		8FZn	1.71 ± 0.06 e	2.42 ± 0.07 ab	*2.06 ± 0.10 Y*
		8FZn+10FSe	1.54 ± 0.08 e	2.55 ± 0.08 a	*2.05 ± 0.14 Y*
	Average		*1.85 ± 0.06 B*	*2.46 ± 0.05 A*	
Ca (g kg^{-1})	Zn soil application	0SZn	8.90 ± 0.32 z	9.17 ± 0.37 z	*9.03 ± 0.24 α*
		50SZn	7.60 ± 0.27 y	9.49 ± 0.32 z	*8.55 ± 0.27 β*
	Foliar application	0F	8.84 ± 0.32 ab	8.63 ± 0.26 bc	*8.74 ± 0.20 Y*
		10FSe	9.56 ± 0.35 ab	9.53 ± 0.72 ab	*9.55 ± 0.39 Z*
		8FZn	7.64 ± 0.20 cd	9.23 ± 0.40 ab	*8.44 ± 0.30 Y*
		8FZn+10FSe	6.96 ± 0.37 d	9.92 ± 0.41 a	*8.44 ± 0.47 Y*
	Average		*8.25 ± 0.24*	*9.33 ± 0.24*	
Fe (mg kg^{-1})	Foliar application	0F	221.6 ± 32.9	76.1 ± 4.0	*148.9 ± 24.7 ZY*
		10FSe	230.4 ± 28.9	86.9 ± 7.7	*158.7 ± 23.5 Z*
		8FZn	156.9 ± 12.1	72.3 ± 5.8	*114.6 ± 12.7 XY*
		8FZn+10FSe	128.8 ± 12.6	71.4 ± 4.7	*100.1 ± 9.9 X*
	Average		*184.4 ± 13.6 A*	*76.7 ± 2.9 B*	

In the case of Fe concentration, although the LSD test was performed on the transformed variable, back-transformed values are presented to ease interpretation

average 22.7 mg kg^{-1} DM and 28.6 μg Se kg^{-1} DM, respectively, in both cases under the threshold of the recommended values for livestock (Suttle 2010). Therefore, the soil conditions found in the present study might provide a very appropriate framework to evaluate the suitability of forage peas to be included in biofortification programs, proposed in this case with Se and Zn. Thus, the present study might provide a very reliable insight into the effectiveness of the biofortification with Zn and Se in areas with low soil availability, which are quite frequent in Spain (Poblaciones et al. 2013; Gomez-Coronado et al. 2016) and other parts of the world (Arthur 2003; Alloway 2009), presenting then a very broad application range.

Besides the pursued objective of increasing the Zn and Se concentrations in the edible part of crops, the idea of a combined application of those minerals was also to reduce application costs for farmers under rainfed extensive cropping systems where gains are usually not very above the profitability threshold. As well with this purpose, the present study was designed to perform the soil Zn application only once at the beginning of the experiment and with the minimum amount possible, in order to cheapen the total inputs as this application might be the most costly, with the premise that such an amount might be enough to satisfy the crop requirements and that residual effect could persist at least for the following cropping year. The analyses of the extractable Zn into soil along the two experimental years showed that after the soil application, the Zn concentration in soil increased up to 1.22 mg kg^{-1} on average, remaining always above 0.5 mg kg^{-1}, critical value to meet the crop needs according to Sims and Johnson (1991). This fact confirmed then the assumption that the used soil fertilization rate was high enough to reach the values of available Zn into soil above the crop requirements in both the application year and at least in the following cropping year. This result was in agreement with that stated in previous studies in other crops such as maize (Karimian and Yasrebi 1995; Shaver et al. 2007), where an important Zn residual effect into soil after a Zn sulfate fertilizer application was reported.

The environmental conditions of the study year had a key importance in the present study by affecting directly most of the parameters analyzed in forage or by influencing the

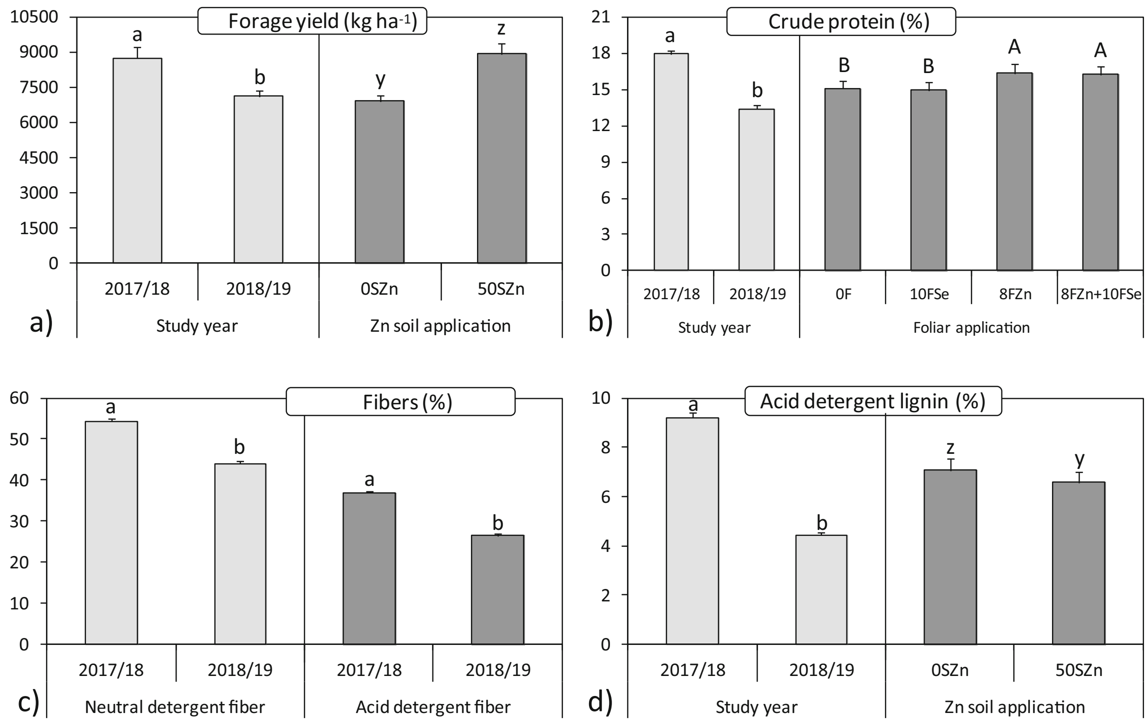

Fig. 5 Influence of main effects **a** study year and Zn soil application on forage yield, **b** study year and foliar application on crude protein, **c** study year on neutral and acid detergent fiber, and **d** study year and Zn soil application on acid detergent lignin. Charts indicate means ($n = 4$), and error bars indicate standard error. Within each parameter and factor, different letters mean significant differences between means according to the LSD test ($P \leq 0.05$). In order to make the differences clearer, a different set of letters was assigned to each factor (lowercase letters [a, b] for study year, lowercase letters [z, y] for Zn soil application, and uppercase letters for foliar application). In the case of forage yield, although the LSD test was performed on the transformed variable, back-transformed values are presented to ease interpretation

effectiveness of the evaluated biofortification with Zn and Se. This influence could be mainly attributed to the very important differences between years in the climatic conditions, characteristic of Mediterranean climate, which are known to play a major role in the growth and performance of crops, especially precipitation in a rainfed cropping system. In this respect, the rainfall distribution pattern was completely different between the study years, and both years were quite different to the average year. the first year (2017/2018) showed a higher than usual rainfall, especially in spring, while the second year (2018/2019) was extremely dry, accounting a rainfall around 35% lower than normal, with important drought periods during February and March. The direct effect of the climatic conditions, especially rainfall, on the growth, performance, grain and forage yield, and nutritive value parameters has already been the object of multiple studies in many crops, such as those of Páscoa et al. (2017). Therefore, no further discussion is going to be made here about the climatic influence, other than that affecting the effects of the Zn and Se application, main objective of the present study. Even though, to consider the dilution effect caused by the different forage yield between growing seasons, the total content of Zn and Se per ha was also determined. However, as discussed later, not very different results to those obtained with the Zn and Se concentrations were observed.

Forage pea has demonstrated a great capacity to increase Zn and Se accumulation in the forage after the foliar application, although in the case of Zn, the efficiency depended on the specific conditions of the study year. Thus, the foliar application of 8 kg $ZnSO_4$-$7H_2O$ ha^{-1} sprayed in two doses of 4 kg ha^{-1} each produced a 4-fold increase in the Zn concentration of forage in 2017/2018 and a 2.8-fold increase in 2018/2019. Therefore, considering that the study year 2017/2018 was much more rainy than 2018/2019, it can be hypothesized that water availability might increase the uptake and accumulation of Zn. Adequate soil moisture has been shown to increase the Zn uptake through roots by facilitating diffusion (Moraghan and Mascagni Jr 1991). However, in this case, as the application was foliar, the higher accumulation in the most humid year could be related to the greater vegetative volume of the crop which could absorb a higher amount of the fertilizer in the moment of the application. This consideration might also explain the similar results obtained when the total Zn content per ha was considered instead of Zn concentration in forage. This fact, which might allow discarding a dilution effect, could mean that Zn absorption might be more dependent on the amount of leaf area per ha, rather than the amount of Zn applied, as long as the Zn applied is enough. The absorption of nutrients by leaves has been proposed to occur mainly through the cuticle, stomata, and/or trichomes (Fernández et al. 2013).

In some other crops, such as soybean, tomato, or sunflower, cuticle and trichomes seem to be the main pathways of Zn absorption (Li et al. 2018, 2019). According to that and considering the absence of trichomes in pea leaves (Villani and Demason 1999), cuticle might be the relevant tissue related to the absorption for this crop. However, as it was not specifically studied in the present research, further research should be performed to confirm it. In any case, those conditions that result in a larger leaf area may produce a higher number of stomata, trichomes, or cuticle surface per ha, and consequently may lead a higher Zn absorption.

Although the Zn accumulation was higher in the most favorable conditions for plant development, even in a very dry year such as 2018/2019, the foliar Zn application produced forage with a Zn concentration able to meet widely the livestock requirements, regardless the livestock type (Suttle 2010). Therefore, the Zn biofortification by means of foliar applications on forage peas might be perfectly suitable under semiarid conditions, where the climatology is very variable between years. The soil Zn application might be convenient to increase the forage yield of the crop as long as the soil concentration is deficient such as in our conditions. However, this soil Zn application did not increase the Zn concentration in forage, which is in disagreement with those observed in other legumes, like chickpea (Ullah et al. 2020), where the soil application was the most effective treatment in the Zn enrichment, but in this case of the grain. It is possible that in our case, the amount of soil Zn supplemented might have compensated only partially the initial high Zn deficiency into soil, enough to allow plants increasing their vegetative growth, but not for an extra Zn accumulation in forage. The application of foliar Se did not present any significant incidence in the accumulation of Zn in peas forage, probably because it did not have any influence in the forage production as Se is not considered an essential nutrient to plants.

The biofortification with Se in peas was also quite effective, as the foliar application of 10 g Se ha^{-1} was able to produce more than a 3-fold increase in the Se concentration of forage, up to 95 μg Se kg^{-1}, amount which almost meets the threshold of 100 μg Se kg^{-1} recommended for livestock (Suttle 2010). However, the most interesting result regarding Se accumulation was the important synergic effect with foliar Zn application. Thus, Se accumulation in forage reached a value of almost 150 μg Se kg^{-1}, close to 5-fold increase in relation to the control, when combining foliar Zn and Se treatments. Even in the case of no Se application, when Zn was foliarly applied, Se concentration in forage increased 36% comparing to the controls. This high enhancement or strengthening in the Se accumulation in plant parts caused by the Zn application has already been evidenced for wheat by Germ et al. (2013). These authors proposed that as Se is assimilated in plants via a sulfur assimilation pathway (Broadley et al. 2012), the over-expression of sulfate transporters caused by the application of Zn, which it is already known to happen (Na and Salt 2011), might produce a more efficient Se assimilation. However, the inverse relation did not happen as the application of foliar Se did not increase the accumulation of Zn in forage. Therefore, further studies should be performed to clarify more deeply the mechanisms involved in this interaction. As in the case of Zn, when total Se content per ha was used as response variable instead of Se concentration, results were also quite similar, limiting then the importance of a dilution effect.

In biofortification programs, besides the increase in the total amount of the target nutrients in the edible parts, the enhancement of their bioavailability might be also crucial. In this regard, an important aspect linked with nutrient bioavailability is the phytate concentration because it is a phosphorous-containing compound that reduces the nutrient absorption, especially for Ca, Fe, Mg, and Zn (Gupta et al. 2015). By contrast, in the case of Se, a positive relationship between phytate and Se status has been found in chicks (Shan and Davis 1994). Therefore, to evaluate the effectiveness of biofortification, it is also important to consider how the application of the target nutrients, Se and Zn in this case, may affect the phytate concentration in the edible parts. In the present study, phytate concentration (estimated through phytic acid) was affected by the soil Zn application, although only in 2017/2018, the most rainy year. In this year, the Zn application produced an increase in the phytate concentration, reaching 6.97 g kg^{-1} (vs the 6.89 g kg^{-1} in the no-soil Zn application treatment), values which were very similar to those obtained previously in the grain of peas (Poblaciones and Rengel 2016, 2017). However, the phytate:Zn molar ratio is considered a better indicator of Zn bioavailability than total acidity in the diet (Ghasemi et al. 2013). Ratios greater than 15 were associated with Zn deficiency (Morris and Ellis 1989). According to that, only foliar Zn treatments provided forage with values of molar ratio phytate:Zn lower than 15 (9 on average), regardless the study year and the Se application. Therefore, the application of foliar Zn, besides increasing the Zn concentration in forage, increased its bioavailability. The application of foliar Se did not have any significant effect in the phytate content, as it had also been found in previous studies for other legume crops, such as cowpeas (Silva et al. 2019).

Although the key aspect of the present research was to evaluate the combined biofortification in forage peas in terms of Zn and Se accumulation in forage, the knowledge of how it affects the yield and the main nutritive value characteristics of the forage might also be of great interest. According to that, soil Zn application significantly affected forage yield. The addition of 50 kg $ZnSO_4$-$7H_2O$ ha^{-1} produced an increase of about 28–30%, very similar in both study years, supporting the statement above about the residual effect of Zn into soil. The increase of forage yield when Zn was applied can be explained because Zn is an essential nutrient for plants, involved in many important physiological plant processes such as growth status, protein metabolism, and phytohormone formation (Cakmak et al. 1989). Although peas are considered a crop with a low relative sensitivity to soil Zn deficiency (Alloway 2008), several authors obtained increments in pea yield after Zn application under soil Zn deficiency conditions (Pandey et al. 2013; Poblaciones and Rengel 2016). In the present study, Zn and/or Se foliar application did not affect forage yield, probably due to the low application rate of these foliar treatments and because Se is not considered an essential nutrient to plants. Furthermore, foliar application was performed for both minerals at the start of the flowering stage, when most of the forage biomass had already been developed and the vegetative growth is clearly slowed in favor of flowering, limiting or preventing then any significant incidence in the forage yield during this short time remaining until harvesting. This result was in clear agreement with those obtained in peas by Poblaciones and Rengel (2017) for Zn and Se under greenhouse conditions and Poblaciones et al. (2013) for Se under field conditions.

Regarding the nutritive value parameters in forage, the variable "foliar treatment" did affect the crude protein and ash content. Crude protein in forage was increased by the foliar treatments containing Zn, fact supported by other studies (Pandey et al. 2013; Poblaciones and Rengel 2016). This fact has been associated with the involvement of Zn in protein synthesis, avoiding RNA degradation, decreased activity of RNA polymerase, ribosomal deformation, and a decrease in the number of ribosomes (Cakmak et al. 1989). Foliar Zn fertilization has also been found to increase *Rhizobium* nodulation in other legumes, such as lentils (Singh and Bhatt 2013). The highest N-fixation that a larger nodulation might produce could also explain the higher crude protein content observed after the foliar Zn application. This increase of protein content, besides the already indicated forage yield increase, might be of vital importance to get the involvement of farmers in the implementation of these biofortification programs, as forage peas might be mainly cultivated as a protein resource for animal feeding due to its high content and its excellent quality derived of its amino acid composition (Robinson et al. 2019). Regarding ashes, their content in forage decreased when both foliar Zn and Se were applied. It was also affected by the soil Zn application, increasing its value when Zn was applied but only in the case of 2017/2018, the year with the highest rainfall. This parameter determines the mineral content (the inorganic fraction) of biomass. Therefore, as the most important minerals for animal feeding were also analyzed individually, the interpretation of this fraction is going to be made specifically for each nutrient.

On the other hand, soil Zn application also affected ADL content in forage, increasing its value when soil Zn was applied. Although the difference was very limited, this fact could be considered negative since a nutritive value point of view as lignin is indigestible for both ruminants and non-ruminants, decreasing then the digestibility of forage (Van Soest 1967). As lignin tends to increase throughout the plant life cycle (Santamaria et al. 2014), an acceleration of plant maturity caused by an increase in Zn soil availability could explain these higher lignin values when Zn was applied to soil. Studies of Chen and Ludewig (2018) suggesting a delay in flowering under Zn deficiency may support that explanation, but further experiments including an exhaustive analysis of the exact growth stage of the plant after treatments should be performed in order to confirm this hypothesis.

In relation to the mineral content in forage, soil Zn application affected Mg and Ca concentrations, decreasing their values when Zn was applied but only in 2017/2018, the most humid year. This fact could be explained by a dilution effect caused by the highest forage yield obtained with the Zn application, considering that no application of those mineral was performed in the experiment. This effect only happened in 2017/2018, probably because of the combination of high rainfall and high Zn availability for plants, which resulted in a higher forage yield, multiplying and enhancing such a dilution effect. Nevertheless, those minerals have been shown to be antagonistic between them (René et al. 2017), which could also explain the negative relationship. The concentration of both minerals, Mg and Ca, in forage was also affected by foliar application, tending to decrease when foliar Zn was applied, especially in the most humid year 2017/2018. Several macronutrients, including Ca and Mg, are known to inhibit the Zn absorption (Alloway 2008). As this antagonism might be supposedly bidirectional, the increase in forage of Zn concentration when foliar Zn was applied might explain then the diminution of Ca and Mg. Finally regarding Fe, the foliar applications containing Zn caused a clear decrease in the Fe concentration. Iron-zinc interaction seems to be complex as both positive and negative (even neutral) effects between them can be found in the literature (Saha et al. 2017; Zou et al. 2019). Therefore in general terms, the Zn application had a negative influence in the concentration in forage of Mg, Ca, and Fe, all of them are essential nutrients for livestock, but in any case, even in the more severe diminutions, the concentration levels of those three minerals were above the threshold of recommended values (Suttle 2010). Finally, soil Zn and foliar Zn and/or Se application also affected somehow the phytate:Mg, Ca, and Fe molar ratio, which might affect also their bioavailability, but such effects were very limited.

5 Conclusions

The present study showed the suitability of forage peas to be used for a combined agronomic biofortification of Zn and Se under Mediterranean conditions in order to increase their concentration in forage, alleviating their deficiency in livestock. For biofortification purposes, the sole simultaneous foliar application of Zn and Se, at the flowering stage at a rate of 8 kg zinc sulfate ha^{-1} (sprayed in two doses of 4 kg ha^{-1} each) and 10 g sodium selenate ha^{-1}, was enough to increase the Zn and Se concentration in forage above the recommended values. However, when 50 kg zinc sulfate ha^{-1} was also applied to soil before sowing, besides the Zn and Se accumulation increase, forage yield increased also a 30%, fact of great interest for farmers. Other positive aspects of the combined biofortification were to obtain forage with a higher crude protein content and to enhance Zn bioavailability. The simultaneous Zn and Se application, which might reduce cropping costs for farmers, resulted quite effective and without undesirable antagonistic effects between these two trace elements; conversely, a certain synergistic effect was observed between them.

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References

Adams ML, Lombi E, Zhao FJ, McGrath SP (2002) Evidence of low selenium concentration in UK bread-making wheat grain. J Sci Food Agric 82:1160–1165. https://doi.org/10.1002/jsfa.1167

Allmang C, Krol A (2006) Selenoprotein synthesis: UGA does not end the story. Biochimie 88:1561–1571. https://doi.org/10.1016/j.biochi.2006.04.015

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

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

AOCS (2006) Official methods of analysis. Association of Official Analytical Chemists. AOCS, Washington DC

Arthur JR (2003) Selenium supplementation: does soil supplement help and why? Proc Nutr Soc 62:393–397. https://doi.org/10.1079/PNS2003254

Bremner JM (1996) Nitrogen total. In: Sparks DL (ed) Methods of soil analysis, part 3. Chemical Methods, Madison, pp 1085–1121

Broadley MR, White PJ, Bryson RJ, Meacham MC, Bowen HC, Johnson SE, Hawkesford MJ, McGrath SP, Zhao FJ, Breward N, Harriman M, Tucker M (2006) Biofortification of UK food crops with selenium. Proc Nutr Soc 65:169–181. https://doi.org/10.1079/PNS2006490

Broadley M, Brown P, Cakmak I, Rengel Z, Zhao F (2012) Function of nutrients: micronutrients. In: Marchner P (ed) Marschner's mineral nutrition of higher plants, 3rd edn. Academic Press, London, pp 191–248. https://doi.org/10.1016/B978-0-12-384905-2.00007-8

Cakmak I (2008) Zinc crops 2007: improving crop production and human health. Plant Soil 306:1–2. https://doi.org/10.1007/s11104-008-9584-6

Cakmak I, Marschner H, Bangerth F (1989) Effect of zinc nutritional status on growth, protein metabolism and level of indole-3-acetic acid and other phytohormones in bean (*Phaseolus vulgaris* L.). J Exp Bot 40:405–412. https://doi.org/10.1093/jxb/40.3.405

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

Chen X, Ludewig U (2018) Biomass increase under zinc deficiency caused by delay of early flowering in *Arabidopsis*. J Exp Bot 69:1269–1279. https://doi.org/10.1093/jxb/erx478

Chibani C, Chabaca R, Boulberhane D (2010) Fourrages algériens. 1. Composition chimique et modèles de prédiction de la valeur énergétique et azotée. Livest Res Rural Dev 22:Article #153. Retrieved from http://www.lrrd.org/lrrd22/8/chab22153.htm

Das S, Chaki AK, Hossain A (2019) Breeding and agronomic approaches for the biofortification of zinc in wheat (*Triticum aestivum* L.) to combat zinc deficiency in millions of a population: a Bangladesh perspective. Acta Agrobot 72:1–13. https://doi.org/10.5586/aa.1770

Day PR (1965) Particle fractionation and particle-size analysis. In: Black CA (ed) Methods of soil analysis, part I. Agronomy 9. American Society of Agronomy, Madison, pp 545–566. https://doi.org/10.2134/agronmonogr9.1.c43

Dinh QT, Cui Z, Huang J, Tran T, Wang D, Yang W, Zhou F, Wang M, Yu D, Liang D (2018) Selenium distribution in the Chinese environment and its relationship with human health: a review. Environ Int 112:294–309. https://doi.org/10.1016/j.envint.2017.12.035

Fan Q, Wang Z, Chang S, Peng Z, Wanapat M, Bowatte S, Hou F (2020) Relationship of mineral elements in sheep grazing in the highland agro-ecosystem. Asian-Australas J Anim Sci 33:44–52. https://doi.org/10.5713/ajas.18.0955

Fernández V, Sotiropoulos T, Brown P (2013) Foliar fertilization: scientific principles and field practices. International Fertilizer Industry Association (IFA), Paris

Gawalko E, Garrett RG, Warkentin TD, Wang N, Richter A (2009) Trace elements in Canadian peas: a grain safety assurance perspective. Food Addit Contam 26:1002–1012. https://doi.org/10.1080/02652030902894389

Germ M, Pongrac P, Regvar M, Vogel-Mikuš K, Stibilj V, Jaćimović R, Kreft I (2013) Impact of double Zn and Se biofortification of wheat plants on the element concentrations in the grain. Plant Soil Environ 59:316–321. https://doi.org/10.17221/6/2013-PSE

Ghaderzadeh S, Mirzaei Aghjeh-Gheshlagh F, Nikbin S, Navidshad B (2016) A review on properties of selenium in animal nutrition. Iran J Appl Anim Sci 6:753–761

Ghasemi S, Khoshgoftarmanesh AH, Afyuni M, Hadadzadeh H (2013) The effectiveness of foliar applications of synthesized zinc-amino acid chelates in comparison with zinc sulphate to increase yield and grain nutritional quality of wheat. Eur J Agron 45:68–74. https://doi.org/10.1016/j.eja.2012.10.012

Gomez-Coronado F, Poblaciones MJ, Almeida A, Cakmak I (2016) Zinc (Zn) concentration of bread wheat grown under Mediterranean conditions as affected by genotype and soil/foliar Zn application. Plant Soil 401:331–346. https://doi.org/10.1007/s11104-015-2758-0

Gupta RK, Gangoliya SS, Singh NK (2015) Reduction of phytic acid enhancement of bioavailable micronutrients in food grains. J Food Sci Technol 52:676–684. https://doi.org/10.1007/s13197-013-0978-y

Hazelton P, Murphy B (2016) Interpreting soil test results. What do all the numbers mean? 3th edn. CSIRO Publishing, Collingwood. https://doi.org/10.1111/sum.12402

Hill GM, Shannon MC (2019) Copper and zinc nutritional issues for agricultural animal production. Biol Trace Elem Res 188:148–159. https://doi.org/10.1007/s12011-018-1578-5

Hla Hla E, Tengda Z, Muhammad Umer F, Rui Z, Yang S, Xin H, Yujie Z, Yuanke L, Zhichen T, Ye X, Xiaomei J, Jianqing Z (2019) Evaluation on zinc and selenium nutrients in polished rice of rice genotypes under zinc biofortification. Biomed J Sci Tech Res 21:16205–16212. https://doi.org/10.26717/BJSTR.2019.21.003666

Karimian K, Yasrebi J (1995) Prediction of residual effects of zinc sulfate on growth and zinc uptake of corn plants using three zinc soil tests. Commun Soil Sci Plant Anal 26:277–287. https://doi.org/10.1080/00103629509369296

Li C, Wang P, Lombi E, Cheng M, Tang C, Howard DL, Menzies NW, Kopittke PM (2018) Absorption of foliar applied Zn fertilizers by trichomes in soybean and tomato. J Exp Bot 69:2717–2729. https://doi.org/10.1093/jxb/ery085

Li C, Wang P, Van der Ent A, Cheng M, Jiang H, Read TL, Lombi E, Tang C, de Jonge M, Menzies NW, Kopittke PM (2019) Absorption of foliar-applied Zn in sunflower (*Helianthus annuus*): importance of the cuticle, stomata, and trichomes. Ann Bot 123:57–68. https://doi.org/10.1093/aob/mcy135

Linn JG, Martin NP (1991) Forage quality test and interpretation. Vet Clin North Am: Food Anim Pract 7:509–523. https://doi.org/10.1016/S0749-0720(15)30790-8

Manojlović MS, Lončarić Z, Cabilovski RR, Popović B, Karalić K, Ivezić V, Ademi A, Singh BR (2019) Biofortification of wheat cultivars with selenium. Acta Agric Scand Sect B Soil Plant Sci 69:715–724. https://doi.org/10.1080/09064710.2019.1645204

MAPA (Spanish Ministry of Agriculture, Fishering and Food) (2020) Anuario de estadística alimentaria del 2018. MAPA, Madrid Retrieved from https://www.mapa.gob.es/es/estadistica/temas/publicaciones/anuario-de-estadistica/default.aspx. Accesed 6 Oct 2020

Mehdi Y, Dufrasne I (2016) Selenium in cattle: a review. Molecules 21:545. https://doi.org/10.3390/molecules21040545

Moraghan JT, Mascagni HJ Jr (1991) Environmental and soil factors affecting micronutrient deficiencies and toxicities. In: Mordvedt JJ, Cox FR, Shumann LM, Welch RM (eds) Micronutrients in agriculture. Soil Science Society of America, Madison, pp 371–425. https://doi.org/10.2136/sssabookser4.2ed.c11

Morris ER, Ellis R (1989) Usefulness of the dietary phytic acid/zinc molar ratio as an index of zinc bioavailability to rats and humans. Biol Trace Elem Res 19:107–117. https://doi.org/10.1007/BF02925452

Na GN, Salt DE (2011) The role of sulfur assimilation and sulfur-containing compounds in trace element homeostasis in plants. Environ Exp Bot 72:18–25. https://doi.org/10.1016/j.envexpbot.2010.04.004

Novoselec J, Klir Ž, Domaćinović M, Lončarić Z, Antunović Z (2018) Biofortification of feedstuffs with microelements in animal nutrition. Agric 24:25–34. https://doi.org/10.18047/poljo.24.1.4

Oropeza-Moe M, Wisløff H, Bernhoft A (2015) Selenium deficiency associated porcine and human cardiomyopathies. J Trace Elem Med Biol 31:148–156. https://doi.org/10.1016/j.jtemb.2014.09.011

Pandey N, Gupta B, Pathak GC (2013) Enhanced yield and nutritional enrichment of seeds of *Pisum sativum* L. through foliar application of zinc. Sci Hortic 164:474–483. https://doi.org/10.1016/j.scienta.2013.10.013

Páscoa P, Gouveia CM, Russo A, Trigo RM (2017) The role of drought on wheat yield interannual variability in the Iberian Peninsula from 1929 to 2012. Int J Biometeorol 61:439–451. https://doi.org/10.1007/s00484-016-1224-x

Petković K, Manojlović M, Čabilovski R, Krstić Đ, Lončarić Z, Lombnæs P (2019) Foliar application of selenium, zinc and copper in alfalfa (*Medicago sativa* L.) biofortification. Turkish J Field Crop 24:81–90. https://doi.org/10.17557/tjfc.569363

Poblaciones MJ, Rengel Z (2016) Soil and foliar zinc biofortification in field pea (*Pisum sativum* L.): grain accumulation and bioavailability in raw and cooked grains. Food Chem 212:427–433. https://doi.org/10.1016/j.foodchem.2016.05.189

Poblaciones MJ, Rengel Z (2017) Combined foliar selenium and zinc biofortification in field pea (*Pisum sativum*): accumulation and bioavailability in raw and cooked grains. Crop Pasture Sci 68:265–271. https://doi.org/10.1071/CP17082

Poblaciones MJ, Rengel Z (2018) The effect of processing on *Pisum sativum* L. biofortified with sodium selenate. J Plant Nutr Soil Sci 181:932–937. https://doi.org/10.1002/jpln.201800251

Poblaciones MJ, Rodrigo S, Santamaria O (2013) Evaluation of the potential of peas (*Pisum sativum* L.) to be used in selenium biofortification programs under Mediterranean conditions. Biol Trace Elem Res 151:132–137. https://doi.org/10.1007/s12011-012-9539-x

Poblaciones MJ, Rodrigo S, Santamaria O, Chen Y, McGrath SP (2014) Selenium accumulation and speciation in biofortified chickpea (*Cicer arietinum* L.) under Mediterranean conditions. J Sci Food Agric 94:1101–1106. https://doi.org/10.1002/jsfa.6372

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

Reich HJ, Hondal RJ (2016) Why nature chose selenium. ACS Chem Biol 11:821–841. https://doi.org/10.1007/s42729-020-00216-y

René PJJR, Heinen M, Dimkpa CO, Bindraban PS (2017) Effects of nutrient antagonism and synergism on yield and fertilizer use efficiency. Commun Soil Sci Plant Anal 48:1895–1920. https://doi.org/10.1080/00103624.2017.1407429

Robinson GHJ, Balk J, Domoney C (2019) Improving pulse crops as a source of protein, starch and micronutrients. Nutr Bull 44:202–215. https://doi.org/10.1111/nbu.12399

Rodrigo S, Santamaria O, Lopez-Bellido FJ, Poblaciones MJ (2013) Agronomic selenium biofortification of two-rowed barley under Mediterranean conditions. Plant Soil Environ 59:115–120. https://doi.org/10.17221/691/2012-PSE

Rodrigo S, Santamaria O, Poblaciones MJ (2014) Selenium application timing: influence in wheat grain and flour selenium accumulation under Mediterranean conditions. J Agric Sci 6:23–30. https://doi.org/10.5539/jas.v6n3p23

Saha S, Chakraborty M, Padhan D, Saha B, Murmu S, Batabyal K, Seth A, Hazra GC, Mandal B, Bell RW (2017) Agronomic biofortification of zinc in rice: influence of cultivars and zinc

application methods on grain yield and zinc bioavailability. Field Crop Res 210:52–60. https://doi.org/10.1016/j.fcr.2017.05.023
Santamaria O, Rodrigo S, Poblaciones MJ, Olea L (2014) Fertilizer application (P, K, S, Ca and Mg) on pasture in calcareous dehesas: effects on herbage yield, botanical composition and nutritive value. Plant Soil Environ 60:303–308. https://doi.org/10.17221/114/2014-PSE
Shan AS, Davis RH (1994) Effect of dietary phytate on growth and selenium status of chicks fed selenite or selenomethionine. Br Poult Sci 35:725–741. https://doi.org/10.1080/00071669408417738
Shaver TM, Westfall DG, Ronaghi M (2007) Zinc fertilizer solubility and its effects on zinc bioavailability over time. J Plant Nutr 30:123–133. https://doi.org/10.1080/01904160601055145
Silva VM, Boleta EHM, Martins JT, dos Santos FLM, da Rocha Silva AC, Alcock TD, Wilson L, de Sá ME, Young SD, Broadley MR, White PJ, dos Reis AR (2019) Agronomic biofortification of cowpea with selenium: effects of selenate and selenite applications on selenium and phytate concentrations in seeds. J Sci Food Agric 99: 5969–5983. https://doi.org/10.1002/jsfa.9872
Sims JT, Johnson GV (1991) Micronutrient soil tests. In: Mortvedt JJ, Cox FR, Shuman LM, Welch MR (eds) Micronutrients in agriculture (Book series 4). Ed. SSSA, Madison, pp 427–476. https://doi.org/10.2136/sssabookser4.2ed
Singh AK, Bhatt BP (2013) Effects of foliar application of zinc on growth and seed yield of late-sown lentil. Indian J Agril Sci 83:622–626
Sosulski FW, Imafidon GI (1990) Amino acid composition and nitrogen-to-protein conversion factors for animal and plant foods. J Agric Food Chem 38:1351–1356. https://doi.org/10.1021/jf00096a011
Suttle NF (2010) Mineral nutrition of livestock, 4th edn. CABI, Cambridge. https://doi.org/10.1079/9781845934729.0000
Thavarajah D, Thavarajah P, Sarker A, Vandernberg A (2009) Lentils (*Lens culinaris* Medikus subs. *culinaris*): a whole food for increased iron and zinc intake. J Agric Food Chem 57:5413–5419. https://doi.org/10.1021/jf900786e
Thavarajah D, Warkentin T, Vandenberg A (2010) Natural enrichment of selenium in Saskatchewan field peas (*Pisum sativum* L.). Can J Plant Sci 90:383–389. https://doi.org/10.4141/CJPS09154
Ullah A, Farooq M, Nadeem F, Rehman A, Hussain M, Nawaz A Naveed M (2020) Zinc application in combination with zinc solubilizing *Enterobacter* sp. MN17 improved productivity, profitability, zinc efficiency, and quality of Desi chickpea. J Soil Sci Plant Nutr In press: https://doi.org/10.1007/s42729-020-00281-3
USDA United States Department of Agriculture (1998) Soil taxonomy, keys to soil taxonomy, 11th edn. USDA-Natural Resources Conservation Service, Washington DC
Van Soest PJ (1967) Development of a comprehensive system of feed analyses and its application to forages. J Anim Sci 26:119–128. https://doi.org/10.2527/jas1967.261119x
Villani PJ, Demason DA (1999) The Af gene regulates timing and direction of major developmental events during leaf morphogenesis in garden pea (*Pisum sativum*). Ann Bot 83:117–128. https://doi.org/10.1006/anbo.1998.0805
Walkley A, Black IA (1934) An examination of the Degtjareff method for determining organic carbon in soils: effect of variations in digestion conditions and of inorganic soil constituents. Soil Sci 63:251–263. https://doi.org/10.1097/00010694-194704000-00001
White PJ, Broadley MR (2005) Biofortifying crops with essential mineral elements. Trends Plant Sci 10:586–593. https://doi.org/10.1016/j.tplants.2005.10.001
Zhao FJ, McGrath SP (1994) Extractable sulphate and organic sulphur in soils and their availability to plants. Plant Soil 164:243–250. https://doi.org/10.1007/BF00010076
Zou C, Du Y, Rashid A, Ram H, Savasli E, Pieterse PJ, Ortiz-Monasterio I, Yazici A, Kaur C, Mahmood K, Singh S, Le Roux MR, Kuang W, Onder O, Kalayci M, Cakmak I (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