Assessment of Phytotoxicity and Behavior of 1-Year-Aged Zn in Soil from ZnO Nanoparticles, Bulk ZnO, and Zn Sulfate in Different Soil-Plant Cropping Systems: from Biofortification to Toxicity
Journal of Soil Science and Plant Nutrition. 22 (1): 150-164 - 2022-03-01 22(1),
DOI: 10.1007/s42729-021-00640-8
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Assessment of phytotoxicity and behaviour of one-year aged Zn in soil from ZnO nanoparticles, bulk ZnO and Zn sulphate in different soil-plant cropping systems: from biofortification to toxicity

Abstracts

Purpose

This study evaluated the impact of aged ZnO NPs on soil-plant systems compared to bulk ZnO and $ZnSO_4$.

Methods

Green pea and beetroot were grown under greenhouse conditions in two agricultural soils to which 20 mg Zn kg^{-1} and 225 mg Zn kg^{-1} had been applied one year before to a previous crop.

Results

At the high dose, differences in soil extractable Zn ($CaCl_2$ and diethylene triaminepentaacetic acid (DTPA)) were observed between Zn sources. Both ZnO applications and $ZnSO_4$ showed different trends in extractability over time suggesting dissolution of ZnO during crop growth.

Plants accumulated large amounts of Zn in their aboveground parts with root-to-stem transfer factors of up to 5.4. Under acidic conditions, beet plants did not survive the high dose, while pea plants showed a dramatic decrease in growth (85%) and grain yield (96%). ZnO NPs differentially affected Zn accumulation and distribution within the plant tissues, but not plant growth.

Conclusions

Weathered ZnO NPs did not seem to pose any greater potential risk to the environment than their Zn counterparts.

Keywords: weathered ZnO nanoparticles, plant effects, Zn accumulation, plant life cycle, green pea (*Pisum sativum*), beetroot (*Beta vulgaris* L.)

INTRODUCTION

Zinc (Zn) is a micronutrient required for the proper development of plants and is involved in many processes, such as the synthesis of enzymes and carbohydrates, the biosynthesis of chlorophylls, and the regulation of growth hormones. Nanofertilizers such as zinc oxide nanoparticles (ZnO NPs) can contribute to increase crop yield and reduce nutrient loss (Raliya et al. 2018; Sanzari et al. 2019; Zulfiqar et al. 2019). Particles at the nanoscale have different properties of their bulk material (micrometric particle size). These differences can affect the behaviour and transport of metal-based NPs and also their role in promoting plant nutrition or phytotoxicity (Bandyopadhyay et al. 2015; Du et al. 2019). Applications of nanotechnology in agriculture require an evaluation of nanoparticle-plant interactions under relevant experimental conditions (Sanzari et al. 2019). Reported studies have highlighted conflicting results regarding the beneficial or detrimental effects of applying ZnO NPs to plants in soil culture experiments (Pradhan and Mailapalli 2017). Several studies have demonstrated that applying ZnO NPs to soils can have positive effects on plants at the physiological, morphological, biochemical and molecular levels. For example, ZnO NPs enhanced photosynthesis and biomass in lettuce (Xu et al. 2018) and crop production (grain yield) and plant dry biomass in pearl millet (Tarafdar et al. 2014). Likewise, ZnO NPs can promote plant growth and development in tomato plants (Raliya et al. 2015). On the other hand, excessive applications of ZnO NPs had inhibitory effects on seed germination and the plant biomass in several plant species (Bandyopadhyay et al. 2015; Garcia-Gomez et al. 2018a; Yoon et al. 2014). In general, both the positive and negative effects of ZnO NPs on plant growth, yield and physiology depend on application rates (Du et al. 2019; Liu et al. 2015). Different studies indicate that low concentrations of ZnO NPs have beneficial effects on plant growth, whereas concentrations equal to or above 200 mg Zn kg^{-1} may have detrimental effects.

Soil is the main sink for ZnO NPs intentionally applied to agricultural systems, whether as fertilizers or sewage sludge. NPs can also reach the soil via unintentional releases during their life cycle (Rajput et al. 2020). The high reactivity of metallic NPs could differentially affect their soil behaviour, with respect to other counterparts, during ageing. The interactions of ZnO NPs with different soil components lead to chemical and biological reactions and also to physical processes; these may affect the fate, transport and behaviour of NPs. The most important form of

114 transformation, especially in acidic soils, is dissolution, which is highly dependent on soil
115 properties. In addition, ZnO NPs suffer changes in their soil distribution that affect their mobility
116 within soil-plant systems and, as a result, in their availability and toxicity (Dimkpa et al. 2018;
117 Garcia-Gomez et al. 2020). The rate and mechanisms of the weathering process are influenced by
118 physicochemical properties of the soil (Lock and Janssen 2003; Lv et al. 2019; Rutkowska et al.
119 2015).
120 The accumulation of metals in plants and the potential risk of them being transported to
121 groundwater is highly dependent on their soil availability (Rodrigues et al. 2016). One-step
122 chemical extraction, with different extractants such as neutral salts and complexing agents, is
123 routinely used for assessing the trace element contents available to plants in soils. These
124 techniques are also applied to soils spiked with metallic NPs to evaluate the availability of metal
125 to plants and its leaching to the groundwater (Almendros et al. 2020; Dimkpa et al. 2018; Gao et
126 al. 2017; Liu et al. 2015).
127 Most plant studies involving ZnO NPs have been conducted with short exposure times and high
128 doses in non-agricultural soils. However, there are some examples of whole life cycle studies
129 conducted in realistic agricultural scenarios (Akanbi-Gada et al. 2019; García-Gómez et al. 2017;
130 Yoon et al. 2014), although most of these focused on pristine ZnO NPs. The behaviour and effects
131 to plants of ZnO NP transformation products compared to the conventional Zn sources (Dimkpa
132 et al. 2018; Garcia-Gomez et al. 2018b) have hardly been addressed. In relation to this, the main
133 goal of the present research was to study plant responses to weathered ZnO NPs in two different
134 crops: green pea (*Pisum sativum* L.) and beet-root (*Beta vulgaris* L.). With this aim, a long-term
135 greenhouse experiment was performed in lysimeters under semi-realistic agricultural conditions
136 throughout the whole agricultural cycle of two successive crops (cherry tomato/green pea and
137 common bean/beet) grown in two different soils with contrasting physico-chemical properties,
138 especially regarding their pHs (acidic vs. calcareous). Both agronomical (20 mg Zn kg^{-1}) and
139 potentially toxic (225 mg Zn kg^{-1}) doses of Zn from ZnO NPs, bulk ZnO and Zn salt ($ZnSO_4$)
140 were applied to the first crop (cherry tomato or common bean). These doses were selected to
141 evaluate their effectiveness as both fertilizers and sources of phytotoxicity. One year later, a

second crop (green pea or beet) was cultivated in the same lysimeters without any new application of Zn. The parameters studied were: Zn acquisition and its distribution within the different plant parts of green pea and beet plants, Zn leaching, and the potential availability of Zn after its ageing in soils. The fate and effects of weathered Zn in soil from $ZnSO_4$ and bulk ZnO were also evaluated for the two plant species to determine additional risks due to NP properties.

MATERIALS AND METHODS

Nanoparticles characterization

ZnO NPs (<100 nm) were obtained from Sigma-Aldrich (Germany) with a nominal primary particle size of less than 100 nm (i.e., rp≤50 nm). The bulk ZnO form was purchased from Sigma–Aldrich (Germany), and the $ZnSO_4.7H_2O$ was from Panreac (Spain). The size and shape of nanoparticles were determined at the Centro Nacional de Microscopía Electrónica (ICTS) by transmission electron microscopy (TEM) using a JEM-1400 PLUS. The mean size and SD were calculated by observing 200 ZnO NPs in random view fields. The TEM images showed ZnO NPs to be elongated in shape and often linked to aggregates. The size (mean ± SD) of the NPs was 58 ± 30 nm where the longer dimension was measured, with sizes between 20 nm and 100 nm.

Soils Characterization

The soils (an acidic soil and a calcareous soils) were collected a year before conducting this study from the top 20 cm of two agricultural fields at Madrid (Central Spain). Both soils were commonly used to growth cereals. Soils were air-dried and passed through a 2 mm sieve for chemical analysis and the greenhouse experiment. Table 1 details the main physical and chemical properties of the two soils. The acidic soil was classified as silt loam (USDA) and the calcareous soil as silty clay loam (USDA). Both soils had low organic matter contents (< 2%).

Long-term greenhouse experiment

Soil treatments and soil ageing

169 The experiment was conducted in polyethylene lysimeters (24 cm mean internal diameter x 24
170 cm height) in a greenhouse with conditions that approach environmentally realistic conditions.
171 The soils were spiked with ZnO powder (as NPs or at micrometric size) or with aqueous solution
172 of $ZnSO_4$ at average 20 or 225 mg kg^{-1} (Zn basis). Soil applications of 20 mg Zn kg^{-1} were taken
173 as the typical rate recommended to farmers to supplement Zn crop nutrition, applied as ZnO bulk.
174 The concentration of 225 mg Zn kg^{-1} was selected according to previously reported phytotoxicity
175 data of ZnO NPs (Bandyopadhyay et al. 2015; Zuverza-Mena et al. 2017). The soil Zn
176 applications (i.e., 200 and 2250 mg Zn pot^{-1}, respectively) were applied to the soil surface and
177 were mixed with the upper layer of the soils (3 kg of a total of 10 kg, i.e. approximately placed
178 throughout the 0-8 cm soil depth) in the pots, simulating broadcasting and mixing in the topsoil
179 before crop growth, which is a common practice for the application of agricultural products to
180 soils. Therefore, the values of total Zn concentration in the upper soil layer were initially higher
181 (ratio 10:3) than the average Zn application rate in the pot. The control treatment and the Zn
182 treatments were replicated three times for a total of 84 lysimeters (10 kg of air-dried soil per
183 lysimeter). Beans (*Phaseolus vulgaris* L. cv. Contender) and cherry tomatoes (*Solanum*
184 *lycopersicum* L. cv. Cerasiforme) were sown in the lysimeters and grown until maturity in a
185 greenhouse at the Technical University of Madrid (UPM) with no humidity or temperature
186 control. The conditions of this experiment were described in García-Gómez et al. (2017).
187 Following the harvest of the tomato and bean plants, the soils were weathered for 6 months (from
188 August to February) in preparation for reuse in the current experiment.
189
190 Greenhouse experiment
191 In February of the second year, the soils were removed from each lysimeter, homogenized and
192 added back into the lysimeters. Washed gravel (1.5 cm layer) was placed at the bottom of each
193 lysimeter to allow aeration and drainage. The soils were amended with 100, 50 and 125 mg of the
194 macronutrients N, P and K respectively per kg of soil. At the initio of the second crop, the total
195 Zn concentrations in the soils were 40.6 ± 0.9 and 62.7±0.9 in the control soils, 62.6±0.6 and
196 81±3 in the soils treated with 20 mg Zn kg^{-1}, and 265±2 and 290±3 mg Zn kg^{-1} in those treated

with 225 mg Zn kg^{-1}, for the acidic and calcareous soil, respectively. Without any new applications of Zn, green pea (*Pisum sativum* L. cv. Negret) and beetroot (*Beta vulgaris* L. cv. Detroit) were grown in the same lysimeters in which the tomato and common beans had respectively been grown, until their edible parts reached maturity, 60 days for pea and 90 days for beet after emergence. The temperature ranged from 3°C (night) to 38°C (day) and the relative air humidity was between 35% to 85%. Lysimeters were watered during the entire plant cycle with tap water. The water volume was slightly above its water-holding capacity in order to obtain five portions of leachate. Pea fruits were periodically collected. After washing in deionized water, their fresh weights were recorded, and they were oven-dried at 60ºC. At the end of the cropping, soil samples were taken from the upper layer of the soil in the lysimeters (approximately 0-8 cm) for chemical analysis. The plants were removed, rinsed with deionized water and divided in roots, leaf stalks (hereinafter referred to as stems) and green leaves for beet plants, and roots, stems and leaves for pea plants. Plant samples were weight to determine the fresh weight. Their roots were successively washed with deionized water and then with 10 mmol L^{-1} tetrasodium ethylenediaminetetraacetate (Na_4EDTA), in an ultrasound-assisted bath at 35 kHz, for 15 min. The last resulting solutions (containing Zn strongly adsorbed on roots, EDTA-Zn) were filtered (no. 41 filter paper, Whatman) and acidified with HNO_3 (Zhou et al. 2011). After that, the beet roots were peeled. Finally, the plant samples were dried in an oven at 60ºC, except the peeled beet roots that were vacuum freeze-dried. The leachates from the lysimeters were collected in association with watering events using a silicone tube connected to a polyethylene bottle (400 mL). The leachate fractions of each lysimeter were filtered (no. 41 filter paper, Whatman), acidified with HNO_3 and Zn content was determined. The total amounts of Zn leached during the assay was the sum of the total Zn content (mg) from the leachate fractions.

Zinc analyses in soils, plants and leachates

Soil (1 g) and plant (0.5 g) samples (roots, stems, leaves and fruits) were digested in Teflon vessels in a sample preparation block system (SPB Probe, Perkin-Elmer) using HNO_3: HCl:double deionized water (1:1:1) to determine total Zn concentration. Two certified reference soils were

used for the validation of the analytical methods: ERM-CC141 and BCR-143R from the Institute for Reference Materials and Measurements of the European Commission. Two one-step chemical extractions: 0.01 M $CaCl_2$ and 5 mM diethylene triaminepentaacetic acid (DTPA) solution were used. For the $CaCl_2$ extraction, 20 mL of 0.01 M $CaCl_2$ solution were added to 2 g of soil sample and the mixture was shaken at room temperature for 2 h (Houba et al. 2000). The DTPA-Zn was extracted by shaking 10 g of dried soil with 20 ml of DTPA extraction solution (5 mM DTPA, 0.01 M $CaCl_2$ and 0.1 M triethanolamine adjusted to pH 7.3 for 2 hours (Lindsay and Norvell 1978). The Zn concentration in all the extracts, including leachates and EDTA-Zn solutions, was quantified using atomic absorption spectrometry (Analyst 700, Perkin Elmer).

Statistical analysis

Data were expressed as the mean ± standard deviation (n=3). Significant differences between the means for the chemical and toxicological data for each application rate: 20 or 225 mg Zn kg^{-1}, were determined by one-way analysis of variance (ANOVA) followed by a Tukey's H.S.D. ($P<0.05$) after the verification of normality and homogeneity of variances. Multifactorial analyses of variance were used to check the influence of the Zn source, type of crop, Zn rate and soil type on Zn extractability in the soil, on Zn accumulation by the plant and on the effects in the plant.

The capacity to translocate Zn between the different plant parts was determined by the transfer factor (TF), which was calculated as the ratios of Zn concentration (based on dry weight) in the different tissues. In beet, the root to stem TF was calculated in relation to the Zn concentration in the peeled roots.

The Pearson correlation analysis of log-transformed data was used to examine the relationship between the Zn concentrations in the soil (total, $CaCl_2$-Zn and DTPA-Zn) and the Zn concentration in the plant parts. This analysis was applied using the data separately for the two soils, for the two crops and also for the different plant tissues. To complete this study, a principal component analyses (PCA) biplot was constructed, which considered two sorts of variables: (i) Zn concentration in the different parts (root, stem, leaf, and edible part) of each plant species and

(ii) the Zn soil concentrations (Total Zn and $CaCl_2$- and DTPA-extractable Zn) and Zn leached amounts. Analysis were conducted using STATGRAPHICS Centurion software, version 17.

RESULTS

Soil extractable Zn concentrations

The amount of extractable Zn was smaller for the $CaCl_2$ than for DTPA method, regardless of the soil type (Figures 1 and 2). However, despite the differences, high correlations were observed between the two (r^2=0.99, P=0.0000) for both crops in the acidic soil. The extractable Zn ($CaCl_2$-Zn and DTPA-Zn) were much higher for the acidic than the calcareous soil. The initial and final Zn concentrations in both extracts (Figure 1) increased with respect to the control soil at 20 and 225 mg Zn kg^{-1} (P=0.0000), in the acidic soil. In the calcareous soil, adding Zn did not significantly increase the initial and final $CaCl_2$-Zn concentrations, compared to the control (Figure 2A). In contrast, the DTPA-Zn (Figure 2B) depended significantly on the Zn application rate (P=0.0000).

DTPA-Zn showed differences depending on the Zn source used in the treatments at 225 mg Zn kg^{-1}. At this application rate, the DTPA-Zn was initially the highest for the $ZnSO_4$ treatments in both crops (P=0.0000) in the acidic soil (Figure 1b). After harvest, these concentrations for the ZnO NP-treated soils were significantly lower than for the other Zn forms in the acidic soil growing beet plants (P=0.0000). The amount of DTPA-Zn in the calcareous soil (Figure 2B) treated with $ZnSO_4$ at 225 mg Zn kg^{-1} was lower than in that measured in ZnO treated soils and was significant for soils in which pea plants were grown (P=0.0000).

The soil Zn extractability evolved in different ways, depending on the soil type, Zn rate and Zn source. At high doses, notable differences were observed between Zn sources. The amount of $CaCl_2$-Zn did not change with time for the acidic soil treated with $ZnSO_4$, but it increased for soils treated with both the ZnO formulations, which was significant for beet (P=0.0118 and P=0.0110 for ZnO NPs and bulk ZnO, respectively). DTPA-Zn increased for the acidic soils (Figure 1b) treated with bulk ZnO at 225 mg Zn kg^{-1} (P=0.0369 and P=0.0198 for beet and pea, respectively) whereas the calcareous soil (Figure 2B) treated with Zn at 225 mg kg^{-1} showed a tendency for

DTPA-Zn to significantly decrease in the soil treated with $ZnSO_4$ for beet (P=0.0099) and pea (P=0.0002).

Leached amounts of Zn

Data about the amount of Zn (mg) leached from the acidic soil at the end of the experiment are shown in Table 2. These amounts depended significantly on the Zn application rate (P=0.0000) and on the Zn source (P=0.0149 and P=0.0019 for beet and pea, respectively). In leachates recovered from soils cropped with both plant species, the amounts of Zn leached were comparable for the control and treatments with application rates of 20 mg Zn kg^{-1}, with the exception of ZnO NPs for pea plants, but notably increased at the 225 mg Zn kg^{-1} application rate, and especially in soils treated with $ZnSO_4$. The amounts of Zn in the leachates were smaller for beet than for pea, with values of 15 to 38 mg and 28 to 62 mg of Zn, respectively, corresponding to the 225 mg Zn kg^{-1} treatments. In the calcareous soil, the amount of Zn leached was negligible (<0.16 mg) and applying Zn did not increase the amount of Zn found in the leachates when this was compared to the control (data no shown).

Adsorption of Zn on the root surface

Zn strongly adsorbed onto superficial root tissue (EDTA-Zn) of beet plants increased when plants were exposed to $ZnSO_4$ at 20 mg Zn kg^{-1} in the acidic soil and at 225 mg Zn kg^{-1} in the calcareous soil (Table 3). In pea, Zn application increased the Zn concentration adsorbed onto roots at 20 and 225 mg Zn kg^{-1} in the acidic soil and at the high concentration in the calcareous soil, with no significant differences between Zn sources. However, the EDTA-Zn was substantially lower in pea plants exposed to $ZnSO_4$ compared to the two oxides in the acidic soil, especially in the treatments at 225 mg Zn kg^{-1}. The differences were not statistically significant, probably due to the large variability observed in the values obtained in plant roots exposed to ZnO NPs. In both soils, the amount of EDTA-Zn from pea roots was approximately 3-times greater than the Zn concentration detected in roots, whereas the Zn concentration in the peel of the beet root was

307 approximately 30-times greater than for EDTA-Zn, determined for the pooled data from all Zn
308 sources and concentrations.
309
310 *Distribution and accumulation of Zn in plant tissues*
311 The Zn concentrations (mg Zn kg^{-1} plant) in dried plant tissues of beet and pea grown in acidic
312 and calcareous soils are shown in Figure 3 and Figure 4, respectively, with low and high
313 application rates provided for comparison. The Zn concentration in plant tissue appeared to be
314 dose-dependent ($P<0.001$ and $P<0.05$, for the acidic and calcareous soils, respectively), and
315 varied with soil type, plant species, plant tissue type and occasionally with Zn source. Soil type
316 was a key factor for the Zn concentrations in the plants. For example, the Zn concentrations in
317 the beet leaves were 42 times greater in plants grown in acidic soil than in those grown in
318 calcareous soil for the 20 mg Zn kg^{-1} application rate. In pea, the mean Zn concentrations for both
319 application rates increased 10 fold in the leaves of plants grown in the acidic soil as opposed to
320 the calcareous soil. Plant species also influenced Zn transport and distribution within the plant.
321 The highest Zn concentration in beet plants (Figures 3 and 4) was measured in plant leaves in the
322 acidic soil and in root peel in the calcareous soil, although the differences between the tissues
323 were less notable in the second case. The maximum Zn concentration registered in pea plants was
324 observed in stem samples in both soils and for all the Zn sources.
325 ZnO NPs differentially affected Zn accumulation, with respect to other counterparts, for pea
326 grown on acidic soil and for both crops in calcareous soil ($P<0.001$). In acidic soil (Figure 3),
327 ZnO NPs produced higher Zn concentrations in pea stem than bulk ZnO and lower than $ZnSO_4$,
328 at 225 mg Zn kg^{-1}. It is notable that the ZnO NP treatment doubled the Zn concentration in the
329 pea pod when compared to bulk ZnO and $ZnSO_4$ applications at 225 mg Zn kg^{-1}. In the calcareous
330 soil (Figure 4), the ZnO NP application caused the lowest Zn accumulation in beet stem at 20 mg
331 Zn kg^{-1}. In pea, differences were observed at high doses, with ZnO NPs causing lower Zn
332 concentrations in stem than bulk ZnO and $ZnSO_4$.
333 At the agronomic dose (20 mg Zn kg^{-1}), Zn application significantly increased Zn content in edible
334 plant tissues of both crops when compared to the control (beet root and leaf and pea grain) in both

soils ($P<0.01$), although concentration values were always lower than 30 mg Zn kg^{-1} in the calcareous soil. Comparing Zn sources, $ZnSO_4$ produced lower Zn concentrations in beet leaf ($P=0.0000$) and higher Zn concentration in pea grain ($P=0.0000$) than both Zn oxides at 20 mg Zn kg^{-1} in the acidic soil. In the calcareous soil, Zn concentrations in root flesh ($P=0.0000$) varied in the order ZnO NPs < bulk ZnO < $ZnSO_4$ at the low application rate.

Differences in Zn distribution between plant species were reflected in the TFs (Supplementary Table S1). The concentration ratio between stem and root (TFstem/root) was highest in pea (3-5-fold) and the concentration ratio between leaf and stem (TFleaf/stem) was highest in beet (3-5-fold), in both soil types. The TFstem/root values were higher than 1, except for beet grown on acid soil, with values up to 5.4 for pea plants grown on acid soil. The TFleaf/stem values ranged from 1.2 to 3.8 and from 0.5 to 0.8 for beet and pea, respectively. In pea, TFgrain/stem diminished compared to the control soil, regardless of the soil type, and always showed values that were lower than 1 in the treated soils. Some differences in transfer factors were observed between Zn sources but they did not show a clear trend.

Correlation between total and soil extractable Zn concentrations and Zn leached and Zn concentration in the different plant parts

A PCA biplot was generated considering as variables the Zn concentration in the different parts of each crop, the Zn soil concentrations and Zn leached amounts (Figure 5). The variance explained by the first two components was 90.36% (x-axis 79.63%, y-axis 10.73%). The PCA biplot showed that Zn concentrations were clustered by rate and soil type. The controls and the lowest rates (20 mg Zn kg^{-1}) in acidic soil and all treatments in calcareous soil were clustered to the left of the x-zero. The rate of 225 mg Zn kg^{-1} in the acidic soil was grouped to the right of the x-axis. The 20 mg Zn kg^{-1} rate in the acidic soil formed separate groups according to crop. In our study, the PCA did not show different clusters of points depending on the different sources (ZnO NPs, bulk ZnO and $ZnSO_4$). This figure showed that the treatment of 225 mg Zn kg^{-1} in the acidic soil had the greatest influence on all the parameters studied, since this was clustered in the right-

363 hand side of the first component whereas the rest of parameters were clustered on the left rectangle
364 of the first component. According to the PCA biplot, all variables: Zn concentration in the
365 different plant tissues,the Zn soil concentration (Total Zn, DTPA-Zn, $CaCl_2$-Zn) and Zn leached
366 were positively correlated with each other. The amounts of Zn extracted from the soil by the Cl_2Ca
367 and DTPA methods appeared close to the Zn concentration in the root, stem, and leaves, and to
368 the amount of Zn in the leachates, indicating a close and positive relationship between them. Total
369 Zn appeared displaced with respect to the other variables on the x-axes, indicating a lower
370 correlation. Likewise, the Cl_2Ca- and DTPA-extractable methods seemed to have a lower
371 predictive capacity for the assessment of Zn concentration in edible part of both crops compared
372 to the rest of plant tissues.
373 The Pearson analysis showed a positive correlation between the amounts of leached Zn and the
374 extractable Zn ($CaCl_2$ and DTPA), in the acidic soil, in comparisons made on a logarithmic basis,
375 although the correlations were not as good for beet (r^2=0.88-0.91, P=0.0000) as they were for pea
376 (r^2=0.95-0.96, P=0.0000). The Zn concentrations held in different parts of the pea plants
377 correlated with the Zn concentrations extracted with $CaCl_2$ and DTPA, in the acidic soil. The
378 regression factors were (r^2=0.99-0.98, P=0.0000) for root, stem and leaf, and slightly lower for
379 pea pod and pea grain (r^2=0.94-0.96, P=0.0000). In the calcareous soil, it was possible to correlate
380 the increased DTPA-Zn with the Zn plant concentration with r^2 values ranging from 0.86 to 0.95
381 (P=0.0000) in beet and from 0.67 to 0.92 in pea (P<0.001). $CaCl_2$-Zn was not included in the
382 analysis, as it tended not to vary between treatments in calcareous soil.
383

384 *Effects of weathered Zn on plant growth*
385 The treatments with low Zn dose did not significantly affect either plant mortality or growth in
386 either of the soil types, compared to the control (data no shown). At the high Zn dose, the effects
387 were significantly greater in the acidic soil than in calcareous one. In the acidic soil, the beet
388 plants exposed to doses of 225 mg Zn kg^{-1} did not survive after 30 days. The pea plants exhibited
389 severe inhibitory effects on their growth, which led to a significant decrease in the wet weights of

all of their plant parts except their roots (Figure 6). In comparison with the control group, the wet weight of the whole pea plant was reduced by 72 to 88% at this high Zn dose and its fruit production was reduced by 86 to 96% for the different Zn sources. The root/shoot weight ratio also significantly increased by between 58 and 74% relative to the control in pea plants ($P=0.0095$). The different Zn sources produced no significantly different effects in this soil type, although higher growth was observed in pea plants exposed to $ZnSO_4$. The wet weight of different parts of pea plants grown in the acidic soil was approximately 2.5 times higher in the $ZnSO_4$ treatment than with ZnO applications. In the calcareous soil, Zn addition at high doses had no effects on beet. On the contrary, stem wet weights decreased significantly ($P=0.0095$) by 38±5 and 50±6%, and plant weights were respectively reduced by 37±5 and 53±7% ($P=0.0301$) in pea plants treated with ZnO NPs and $ZnSO_4$, at the high exposure concentration. Bulk ZnO application had no significant effect on plant growth. The comparison between Zn sources showed significant differences in stem wet weight between applications of bulk ZnO and those of ZnO NPs and $ZnSO_4$ ($P=0.0322$) in the calcareous soil. The reductions in plant growth observed between treated and control plants were not statistically significant when the effects were measured as the dry weight of plants, regardless of the Zn concentration or source.

DISCUSSION

When added to soil, NPs undergo processes such as sorption, dissolution, and aggregation. These transformations depend on the physicochemical properties of the soil and can affect the availability of Zn in the soil (Meesters et al. 2019). As expected, the extractable Zn concentrations were much higher in the acidic soil than in the calcareous soil because soil pH is the major determining factor affecting solubility and Zn distribution in soil (Gupta et al. 2016). Furthermore, the higher percentage of clay in the calcareous than in the acidic soil could also decrease the extractable Zn due to the adsorption of Zn to this soil constituent (Adriano 2001). The extractable Zn was lower for the $CaCl_2$ than for the DTPA method due to the fact that the $CaCl_2$ extracts only dissolved Zn, or small particles including Zn, in pore water (readily available amounts of Zn), whereas DTPA also extracts exchangeable and weakly bound metals. After a previous culture

418 event and subsequent ageing period, $ZnSO_4$ was able to maintain residual Zn in a more labile
419 form than either of the oxides supplied to the acidic soil at the high application rate. This was
420 shown by the highest DTPA-Zn reading at the beginning of this experiment and the lixiviation
421 rate of the weathered Zn in the soil when Zn was added as $ZnSO_4$. Changes on Zn extractability
422 over time varied for the different Zn sources. Different trends were also observed as a function of
423 Zn extracting solution indicating the balanced influence of dissolution and sorption processes
424 controlling extractable Zn with $CaCl_2$ and DTPA, respectively. Unlike $ZnSO_4$, the amount of
425 $CaCl_2$-Zn increased over time in the acidic soil treated with both oxides at high Zn rates. This
426 suggested that dissolution of ZnO took place and that the component metal ions were released
427 during plant growth. In the case of the 225 mg Zn kg^{-1} applications, the Zn concentration in the
428 soil exceeded that associated with the solubility of oxide; Zn^{2+} was therefore able to coexist in the
429 soil with ZnO, despite ageing (Gao et al. 2017; Manzo et al. 2011). The evolution of DTPA-Zn
430 revealed a different behaviour compared to $CaCl_2$ for the different Zn sources. DTPA-Zn
431 increased for bulk ZnO but not for ZnO NPs. Due to their smaller specific surface area,
432 microparticles are characterised by lower solubility compared to NPs, which could have been
433 subsequently reflected in their lower extractability (Josko et al., 2021). However, the opposite
434 was observed. This could have been due to ZnO NPs remaining as aggregates, which would have
435 reduced the surface area and hampered the diffusion of free ions, and consequently limited the
436 dissolution processes (de Santiago-Martin et al. 2016). The decrease of the DTPA-extractable Zn
437 amount in the calcareous soil treated with 225 mg Zn kg^{-1} over time was probably due to Zn
438 adsorption to the carbonate fraction and also to the organic and clay fractions (de Santiago-Martin
439 et al. 2014; Zhao et al. 2012). In calcareous soils, due to the presence of $CaCO_3$ compounds and
440 to soil pH values, ageing processes can be expected to be more pronounced than in soils with a
441 low pH (Lock and Janssen, 2003b). Zhang et al. (2017) studied the effect of soil properties on
442 aging, after water-soluble Zn was added to 23 soils and incubated for 813 days. They also found
443 that ageing was mainly controlled by soil pH. Donner et al. (2012) showed that Zn fixation of
444 soluble added Zn was strongly related to soil pH and ageing time, and relatively unaffected
445 by soil type and mineralogy. Conversely, the greater extractability observed for both oxides

446 compared to $ZnSO_4$, at the end of the experiment, could partially be attributed to root exudates,
447 which may have affected the dissolution of metal oxides from the NPs and bulk material, thereby
448 increasing soluble Zn levels (Anjum et al. 2016; Dimkpa et al. 2013; Lv et al. 2019; Zulfiqar et
449 al. 2019), and this could have helped to mitigate the consequences of Zn adsorption to the soil
450 components. These results would seem to indicate that NPs could serve as reservoirs for continued
451 ion release, but only at high concentrations.
452 The clustering in the PCA biplot showed that the soil and plant parameters studied depended on
453 the applied Zn rate and soil type, and occasionally on the plant species. Zinc concentrations
454 extracted by Cl_2Ca and DTPA methods were better predictors than total Zn soil concentration of
455 leached Zn amount and Zn accumulated by plants in their different tissues, except for Zn in their
456 edible parts, which correlated better with total Zn concentration in soils. Pearson's analysis
457 showed no differences (r and P values) between the predictive abilities of $CaCl_2$ and DTPA for
458 the amount of Zn leached and accumulated in the different plant parts in the acid soil. However,
459 some differences were observed between both extractors (weak vs. strong) in the calcareous soil.
460 The amounts of Zn leached from the calcareous soil were negligible according to the values of
461 Zn extracted with $CaCl_2$, but the DTPA reagent did not offer a very good approach for assaying
462 the Zn soil-water transference in this soil, as previously reported by Almendros et al. (2020). On
463 the contrary, positive and significant relationships were found between extractable DTPA-Zn and
464 Zn concentration in all plant parts in the calcareous soil. This suggested that DTPA was an
465 effective extractant for estimating plant Zn uptake from the different Zn sources and that plants
466 can take up Zn not only from the soil solution but also from exchangeable and weakly bound
467 fractions. Several authors have reported a high correlation between DTPA-Zn and Zn uptake by
468 plants in calcareous soils (Feng et al., 2005; de Santiago-Martin et al., 2014; Almendros et al.,
469 2015). These findings differ from those found in a previous study, conducted with the same Zn
470 sources and soils, but involving other crops and recently added Zn (Almendros et al. 2020). These
471 authors found that the relationships between the amounts of extractable Zn and Zn concentration
472 in different plant parts were better for the $CaCl_2$ reagent than for the DTPA extractant. However,
473 the strength of these correlations depended on plant species, plant part and soil type.

In soils amended with ZnO NPs, roots were the main entry point of the NPs into plants. When ZnO NPs interact with plant roots, differential absorption and adsorption processes may occur (Anjum et al. 2016). Similar to particles, the adsorption of ions onto root surfaces is also possible. This was shown by the fact that EDTA-Zn in beet roots had its maximum values for $ZnSO_4$. The adsorption process was especially important for pea, in which Zn largely adhered to the external parts of plant roots in both types of soil, with much higher EDTA-Zn values than the Zn concentration measured in roots. Determining root Zn concentrations that ignore the Zn concentration adsorbed by roots could overestimate the amount of Zn accumulated in the pea roots.

Differences in the relationship between the amount of EDTA-Zn and root Zn concentration in beet and pea plants may be due to differences in their root physiology and morphology (Su et al. 2019). Both pea and beet are dicotyledonous plants characterized by a taproot with secondary roots growing laterally from it. In our experiment, pea plants had a deep-penetrating root system with a main root branching into lateral roots. Beet is a tuberous plant in which the roots are thick and modified for food storage. These physiological differences could affect the interaction and distribution of metals and NPs in the root of both plants (Ebbs et al., 2016). In addition, plant roots often release exudates to enhance nutrients uptake. The composition and concentration of exudates in the rhizosphere depends on the plant species and may differentially affect the behavior and availability of metals and metal based NPs to plants (Zulfiqar, et al., 2019).

Analysis of Zn concentration in different plant tissues suggested that Zn bioaccumulation was systemic, since Zn concentrations were correlated across all plant parts for all the chemical Zn forms (Priester et al. 2017; Sanzari et al. 2019). The increase in shoot and leaf Zn concentrations with respect to the control indicated that beet and pea were able to translocate large amounts of Zn applied in the three different Zn forms to their aboveground plant parts. The transfer of Zn from root to stem was especially efficient in pea (Supplementary Table S1). This finding agreed with those reported by other authors who found higher Zn accumulations in stem than root in tomato (Raliya et al. 2015) and in soybean plants treated with ZnO (Yoon et al. 2014). Unlike our

results, several authors have reported Zn translocation factors from roots to stems with values lower than 1 for wheat (Garcia-Gomez et al. 2018a), tomato (Akanbi-Gada et al. 2019) and pea (Mukherjee et al. 2014) from freshly added ZnO NPs. In a soil assay with weathered ZnO NPs and Zn ion, Dimkpa et al. (2018) also reported low Zn translocation to wheat stems. According to these same authors, the low TFstem/root could have been due to an overestimation of the Zn root concentration because adsorption into the root surface is usually ignored. In our study, the Zn concentration in roots was only related to the amount of absorbed Zn.

Of the various tissues in which Zn accumulates, the most important for nutritional quality are the edible parts of crops. The use of agronomic strategies to increase the concentration of mineral elements in edible plant parts is commonly known as agronomic biofortification. From this point of view, and despite ageing, Zn applied to the soil at the agronomic dose (20 mg Zn kg^{-1}), with any of the three sources studied, was effective in increasing the Zn content in beet root and in pea grain in the acidic soil. However, in the calcareous soil, which was deficient in Zn, the Zn application at agronomic rate did not achieve a substantial improvement in the Zn concentration in edible tissues, in accordance with the fact that the amount of soluble Zn was very low in this soil. For example, the Zn concentration in pea grain in the calcareous soil treated with Zn at the agronomic dose was lower than the average (40 mg Zn kg^{-1}) of those found in unfertilized soils (Guindon et al., 2021). Biofortification through the application of mineral fertilizers is a sustainable approach to improve the nutritional profile of food crops. This could be used to help address Zn deficiencies in humans but above certain levels, the amount of accumulated Zn can become toxic. Thus, the high accumulation of Zn in the leaves of beet plants (1021±64 mg Zn kg^{-1}) exposed to 20 mg Zn kg^{-1} in acidic soils could be of concern because the consumption of fresh beet leaves in salads is increasing.

At the high application rates, in the acidic soil, the Zn concentrations in the non-edible vegetative parts of crops were very high, but in pea grain the Zn concentration was equal to or lower than 160 mg Zn kg^{-1}. A notable Zn concentration (2075±140 mg Zn kg^{-1}) was recorded in the pods of pea plants exposed to 225 mg kg^{-1} of ZnO NPs, but such a concentration is unlikely to occur under

normal environmental conditions. In beet roots, the peel can act as a barrier and restrict the incorporation of NPs into the edible tissues. In our study, higher Zn accumulations were observed in the peel than in the flesh of beet plants grown in calcareous soil but there were no significant differences between the different Zn sources, which is agreed with Bradfield et al. (2017). The metal accumulation in the peel (mg Zn) represented 25% of the metal accumulated by the root. Peeling off the outer layer of the beet root did not therefore seem to substantially reduce the potential dietary intake of Zn.

Some authors had also reported differential accumulation in different plant tissues according to their Zn sources, although with contrasting results (Du et al. 2019; Garcia-Gomez et al. 2015; Mukherjee et al. 2014). In the present study, there were some significant differences in Zn accumulation and distribution for the three different Zn sources but no clear trend in these measurements was observed. The most notable difference observed was that the accumulation of Zn from ZnO NPs in pod peas grown in the acidic soil was double the values associated with bulk ZnO and $ZnSO_4$ applied at 225 mg kg^{-1}. Some authors have reported that ZnO NPs can be incorporated by roots as NPs (Lin and Xing 2008; Moghaddasi et al. 2017). However, most studies have suggested that Zn uptake from ZnO NPs into plants probably occurs through the uptake of Zn ions that form various zinc complexes in aboveground plants (Du et al. 2019; Hernandez-Viezcas et al. 2013; Priester et al. 2017; Wang et al. 2013). However, the translocation of Zn in its ionic form could not explain the different patterns of accumulation in tissues observed between plants treated with NPs and their counterparts in this study.

Effects of weathered Zn on plant growth depended on soil type and plant species. Earlier studies have shown that when applied at a low dose, Zn could act as a growth promoter, whereas it could also have detrimental effects on plant growth at high doses (Almendros et al. 2015; Du et al. 2019; Liu et al. 2015). The present study showed that residual Zn in soils treated with low doses (20 mg Zn kg^{-1}) did not have either beneficial or toxic effects on plant growth and productivity in either of the soils, regardless of the initial Zn form. In the calcareous soil, the amounts of DTPA-Zn in the untreated soil (0.50 and 0.72 mg kg^{-1} for beet and pea, respectively) were close to the critical soil levels of 0.1-1.0 mg Zn kg^{-1} dry soil for most crops (Alloway 2004; Lindsay and Norvell

556 1978). What is more, the Zn concentrations in both the plant species exposed to untreated
557 calcareous soil were in the range of 15-30 mg Zn kg^{-1} plant which, according to Broadley et al.
558 (2007) and White and Brown (2010) was critical for showing deficiency symptoms. However,
559 no beneficial effects on plant growth due to Zn nutrient supply were observed. This occurred
560 despite the tissue Zn concentration increasing to well-above the critical deficient level when the
561 beet was grown in calcareous soil spiked with 225 mg Zn kg^{-1} and the pea in soil with both 20
562 and 225 mg Zn kg^{-1}.
563 At the high application rate, the impact of residual Zn greatly depended on the soil properties. In
564 the acidic soil, for most of the treatments, the Zn concentrations in the plants exceeded the
565 threshold toxicity limits for beet (100-150 mg Zn kg^{-1} plant) and pea (380-500 mg Zn kg^{-1} plant)
566 described by Macnicol and Beckett (1985). Severe inhibition of plant growth, to 85%, and of
567 grain production, to 96%, for pea in the acidic soil was consistent with the high level of Zn uptake,
568 which exceeded 5000 mg Zn kg^{-1} in pea stem. Zn concentrations in the stems and leaves of beet
569 exposed to 20 mg Zn kg^{-1} in the acidic soil respectively exceeded 2 and 5 times the toxic limits,
570 but no diminished plant growth was observed. In the calcareous soil, despite the fact that the Zn
571 concentrations in the both plant species were far below their toxicity limits, some singular toxic
572 effects on plant growth were observed in pea at 225 mg Zn kg^{-1}. Discrepancies between Zn
573 accumulation and toxicity could be due to differences in intracellular distribution and speciation
574 of Zn in plants. It is assumed that the concentration of a chemical in an organism can explain
575 observed toxicity and that chemical concentration above a certain level is associated with an
576 adverse biological effect. However, uptake does not necessarily mean toxicity as organisms can
577 sequester metals and avoid their toxic effects (Kouhi et al., 2015). For example, Zn deposition in
578 cell vacuoles may be considered as a detoxification mechanism, increasing tolerance to Zn
579 exposure. In addition, other mechanisms such as Zn adsorption in roots can induce structural and
580 functional disorders. Some authors have demonstrated the interaction of ZnO NPs with root
581 nodules of leguminous plants (Bandyopadhyay et al. 2015, Mukherjee et al., 2014), which could
582 affect the nitrogen fixation process and hence plant growth. Therefore, the level of Zn present in
583 plants is not the only determinant of Zn toxicity.

584 ZnO NPs can act as a source of metal ions and their effects can be due to metal released or can
585 be due to specific properties of the NPs. The toxic effects on plant growth and fruit yield on beet
586 as a result of Zn exposure were essentially independent of the Zn treatment applied. Although the
587 differences were not statically significant, the reduction in pea growth induced by aged ZnO NPs
588 or bulk ZnO at the highest exposure concentration was greater than that caused by $ZnSO_4$,
589 especially in plant root. At the dose of 225 mg/kg, the internal Zn concentration in the root was
590 similar for the three Zn sources applied. However, the amount of Zn adsorbed on the root (EDTA-
591 Zn extractable) was substantially lower in plants exposed to $ZnSO_4$ compared to the two oxides
592 (Table 3). The adhesion of ZnO particles to the root surface could negatively affect plant growth
593 and explains the higher wet weight observed in plants growth when the soil was treated with
594 $ZnSO_4$ at 225 Zn mg kg^{-1} (Kouhi et al., 2015; Rizwan et al., 2017). However, this explanation
595 was not verified by any specific experiments.
596 The differences observed in the calcareous soil among Zn sources showed a different trend. In
597 this soil, the observed differences suggested a higher toxicity of ZnO NPs than bulk ZnO and a
598 toxicity similar to that of $ZnSO_4$. Authors have reported the differential toxicity of ZnO NPs
599 compared to their counterparts but with different results. Garcia-Gomez et al. (2015) reported that
600 the inhibition of growth associated with ZnO NPs was similar to that caused by bulk ZnO and
601 less than that associated with the counterpart ion for three plant species (wheat, radish, and vetch)
602 in a natural soil with an application rate of 1000 mg Zn kg^{-1}. Similarly, ZnO NPs showed fewer
603 inhibitory effects on wheat (Du et al. 2019) and alfalfa growth than $ZnSO_4$ (Bandyopadhyay et
604 al. 2015). These authors also found differences between the influence of oxides: ZnO NPs
605 produced reductions in root and shoot biomass, while bulk ZnO acted as a growth promoter. All
606 of these studies were performed with recently added NPs. In a study with weathered and fresh
607 ZnO NPs and Zn ion at a low application dose, Dimkpa et al. (2018) observed an increase in plant
608 height and grain yield in wheat, irrespective of the Zn source.
609
610 **CONCLUSIONS**

The results presented here showed that the fate and plant effects of weathered Zn were mainly dictated by the soil properties and plant species. However, there were some cases of significant differences in soil extractability, plant accumulation and in lesser extent toxicity as a function of chemical form (NP, bulk or ionic), particularly at high doses. These differences in extractability and plant accumulation observed between Zn sources were not reflected in differential positive or detrimental effects on plants. The effects of weathered ZnO NPs on plants seemed to be mostly due to the toxic effects of the Zn^{2+} ion, whereas the nano size of the particles did not play a crucial role. Overall, the results of this study seem to suggest that the dissolution of ZnO NPs was not complete in aged soils and that some of the specific structure of ZnO NPs was maintained in the soil for quite a long period when it was applied at a high dose. After a 1-year aging, ZnO NPs did not apparently either offer a clear advantage or pose a hazard to agriculture or the environment under our experimental conditions. In addition, NPs did not seem to present either a greater advantage or a greater risk to food safety due to Zn accumulation than $ZnSO_4$ and bulk ZnO.

ACKNOWLEDGMENTS

The authors are grateful to Pilar Ortiz and Javier Sanchez for the technical assistance provided. English Language editing was supplied by MJH Translation.

DECLARATIONS

- Ethics approval and consent to participate

Not applicable

- Consent for publication

Not applicable

- Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request

- Competing interests

The authors declare that they have no competing interests

- Funding

This work was supported by the Spanish Ministry of Economy, Industry and Competitiveness (Spanish projects RTA2013-00091-C02-01 and RTA2013-00091-C02-02) and by the Community of Madrid project S2018/BAA-4330.

- Authors' contributions

Conceptualization: AO and DG; Formal analysis and investigation: AO, DG; Validation of results: PA, CG and MF; Writing - original draft preparation: MF; Writing - review and editing: All authors; Funding acquisition: MF and AO. All authors read and approved the final manuscript.

FIGURES CAPTIONS

Fig. 1 Extractable Zn concentration in soil measured by the $CaCl_2$ (a) and DTPA (b) extraction methods at the beginning and at the end of the assay for different Zn treatments and crops in acidic soil. Different letters indicate significant differences ($P<0.05$) between each Zn source (NP, bulk or $ZnSO_4$) and the control soil for the extractable Zn concentration in soil at 20 mg Zn kg^{-1} (lower case letters) and 225 mg Zn kg^{-1} (capital letters), according to the Tukey post hoc test ($P <0.05$); * indicates significant differences ($P<0.05$) between the initial and final extractable Zn concentration in the soil for each treatment, according to the Tukey post hoc test ($P <0.05$); Values are means for three replicates

Note. In acidic soil, beet plants exposed to 225 mg Zn kg-1 barely developed and did not survive after 30 days

659 **Fig. 2** Extractable Zn concentration in soil measured by the $CaCl_2$ (a) and DTPA (b) extraction
660 methods at the beginning and at the end of the assay for different Zn treatments and crops in
661 calcareous soil. Different letters indicate significant differences (P<0.05) between each Zn source
662 (NP, bulk or $ZnSO_4$) and the control soil for the extractable Zn concentration in soil at 20 mg Zn
663 kg^{-1} (lower case letters) and 225 mg Zn kg^{-1} (capital letters), according to the Tukey post hoc test
664 (P <0.05); * indicates significant differences (P<0.05) between the initial and final extractable Zn
665 concentration in the soil for each treatment, according to the Tukey post hoc test (P <0.05); Values
666 are means for three replicates
667 **Fig. 3** Zinc concentration in different tissues of pea and beet plants grown in acidic soils at
668 different Zn treatments after harvest. Different letters indicate significant differences (P<0.05)
669 between each Zn source (NP, bulk or $ZnSO_4$) and the control soil for Zn concentration in each
670 tissue at 20 mg Zn kg^{-1} (lower case letters) and 225 mg Zn kg^{-1} (capital letters), according to the
671 Tukey post hoc test (P <0.05). Values are means for three replicates
672 **Fig. 4** Zinc concentration in different tissues of pea and beet plants grown in calcareous soils at
673 different Zn treatments after harvest. Different letters indicate significant differences (P<0.05)
674 between each Zn source (NP, bulk or $ZnSO_4$) and the control soil for Zn concentration in each
675 tissue at 20 mg Zn kg^{-1} (lower case letters) and 225 mg Zn kg^{-1} (capital letters), according to the
676 Tukey post hoc test (P <0.05). Values are means for three replicates
677 **Fig. 5** Biplot of the first two principal components of the principal component analysis (PCA) of
678 the Zn soil concentrations (Total Zn and DTPA- and $CaCl_2$-extractable Zn) and Zn leached
679 amounts, and the Zn concentration in the different plant tissue. The parameters are represented
680 as: 1A: total Zn concentration; B: $CaCl_2$-Zn (initial); C: $CaCl_2$-Zn (final); D: DTPA-Zn (initial);
681 E: DTPA-Zn (final); F: Zn leached; G: EDTA-Zn; H: Zn in root; I: Zn in stem; J: Zn in leaf; K:
682 Zn in edible part; a: control (acidic and calcareous soils); b: 20 mg Zn/kg (acidic soil; pea); c: 20
683 mg Zn/kg (acidic soil; beet); d: 20 mg Zn/kg (calcareous soil; beet and pea); e: 225 mg Zn/kg
684 (calcareous soil; beet and pea); f: 225 mg Zn/kg (acidic soil; pea)
685 **Fig. 6** Effects on growth of pea plants (root, stem, leaf, aboveground plant and fruit (pod + grain))
686 exposed to 225 mg Zn kg^{-1} soil in acidic and calcareous soils expressed as a percent of weight

687 with respect to the control. * indicates significant differences between each Zn source (NP, bulk
688 or $ZnSO_4$) and the control soil, according to the Tukey post hoc test ($P < 0.05$). ** indicates
689 significant differences between Zn sources (ZnO NPs, bulk ZnO and $ZnSO_4$), according to the
690 Tukey post hoc test ($P < 0.05$). Values are means for three replicates
691

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Declarations

- Ethics approval and consent to participate

Not applicable

- Consent for publication

Not applicable

- Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request

- Competing interests

The authors declare that they have no competing interests

- Funding

This work was supported by the Spanish Ministry of Economy, Industry and Competitiveness (Spanish projects RTA2013-00091-C02-01 and RTA2013-00091-C02-02) and by the Community of Madrid project S2018/BAA-4330.

- Authors' contributions

Conceptualization: AO and DG; Formal analysis and investigation: AO, DG; Validation of results: PA, CG and MF; Writing - original draft preparation: MF; Writing - review and editing: All authors; Funding acquisition: MF and AO. All authors read and approved the final manuscript.

- Acknowledgements

905 The authors are grateful to Pilar Ortiz and Javier Sanchez for the technical assistance
906 provided. English Language editing was supplied by MJH Translation.

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Table 1. Main physicochemical characteristics and Zn soil concentrations (available and total) in acidic and calcareous soils

Soil Type	pHw (1–2.5)	OM (%)	C.E.C (mmol/100 g)	Texture (%)			Zn soil (mg kg^{-1})	
				Sand	Silt	Clay	Total Zn	Available DTPA-Zn
Acidic soil	5.4	1.69	11.4	25	57	18	40.1	2.49
Calcareous soil	8.5	1.13	22.1	17	44	39	61.8	0.3

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Table 2. Data of Zn total (mg) leached during the assay in the acidic soil. Different letters in the same column indicate significant differences among Zn sources (NP, bulk or $ZnSO_4$) and the control for the same plant species at 20 or 225 mg kg^{-1} according to the Tukey post hoc test ($P < 0.05$).

	Beet		**Pea**	
	20 mg Zn kg^{-1}	**225 mg Zn kg^{-1}**	**20 mg Zn kg^{-1}**	**225 mg Zn kg^{-1}**
	P=0.2153	P=0.0076	P=0.0350	P=0.0006
Control	0.15±0.09a		0.12±0.03a	
ZnO NPs	0.2±0.1a	17±7b	0.68±0.08b	28±6b
bulk ZnO	0.2±0.1a	15±7b	0.4±0.3ab	34±8b
$ZnSO_4$	0.4±0.2a	38±5c	0.3±0.2ab	62±7c

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Table 3. Concentrations of the Zn strongly adsorbed (EDTA-Zn) onto superficial root tissues of beet and pea (mg Zn kg^{-1} root) in acidic and calcareous soil after harvest. Different letters in the same column indicate significant differences among Zn sources (NP, bulk or $ZnSO_4$) and the control soil for the same plant species at 20 or 225 mg kg^{-1} according to the Tukey post hoc test ($P < 0.05$).

		Beet[a]		**Pea**	
		20 mg Zn kg^{-1}	**225 mg Zn kg^{-1}**	**20 mg Zn kg^{-1}**	**225 mg Zn kg^{-1}**
Acidic soil		P=0.0050		P=0.0006	P=0.0082
	Control	1.8±0.7a		133±35a	
	ZnO NPs	3.7±0.6a	-	748±148b	4816±1508b
	bulk ZnO	4.7±0.8a	-	758±130b	4496±517b
	$ZnSO_4$	18±8b	-	597±38b	3382±320b
Calcareous soil		P=0.4706	P=0.0448	P=0.6946	P=0.0155
	Control	1.3±0.7a*		47±16a	
	ZnO NPs	0.8±0.1a	2.2±0.1ab	57±7a	181±13b
	bulk ZnO	1.1±0.2a	2.3±0.2ab	82±34a	143±23b
	$ZnSO_4$	1.2±0.2a	3.2±0.3b	50±2a	172±15b

[a] In acidic soil, beet plants exposed to 225 mg Zn kg^{-1} barely developed and did not survive after 30 days.

Figure 1

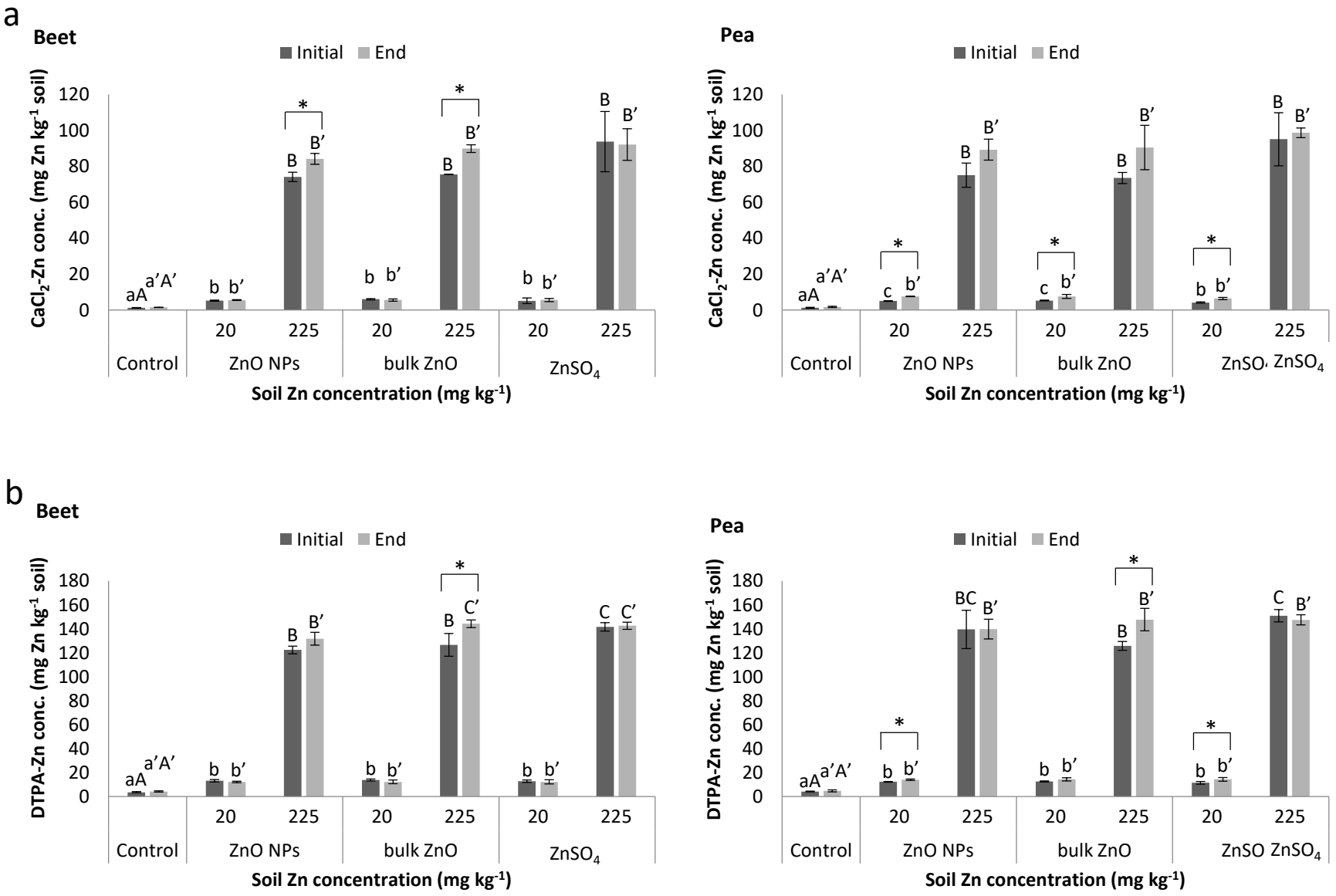

a
Beet
Initial
End
CaCl2-Zn conc. (mg Zn kg-1 soil)
120
100
80
60
40
20
0
aA
a'A'
b b'
B B'
*
20
225
Control
ZnO NPs
bulk ZnO
ZnSO4
Soil Zn concentration (mg kg-1)
Pea
c b'
b b'
b
Initial
End
CaCl2-Zn conc. (mg Zn kg-1 soil)
Control
ZnO NPs
bulk ZnO
ZnSO4
Soil Zn concentration (mg kg-1)
b
Beet
Initial
End
DTPA-Zn conc. (mg Zn kg-1 soil)
180
160
140
120
100
80
60
40
20
0
C C'
Control
ZnO NPs
bulk ZnO
ZnSO4
Soil Zn concentration (mg kg-1)
Pea
BC
Initial
End
DTPA-Zn conc. (mg Zn kg-1 soil)
Control
ZnO NPs
bulk ZnO
ZnSO4
Soil Zn concentration (mg kg-1)

Figure 2

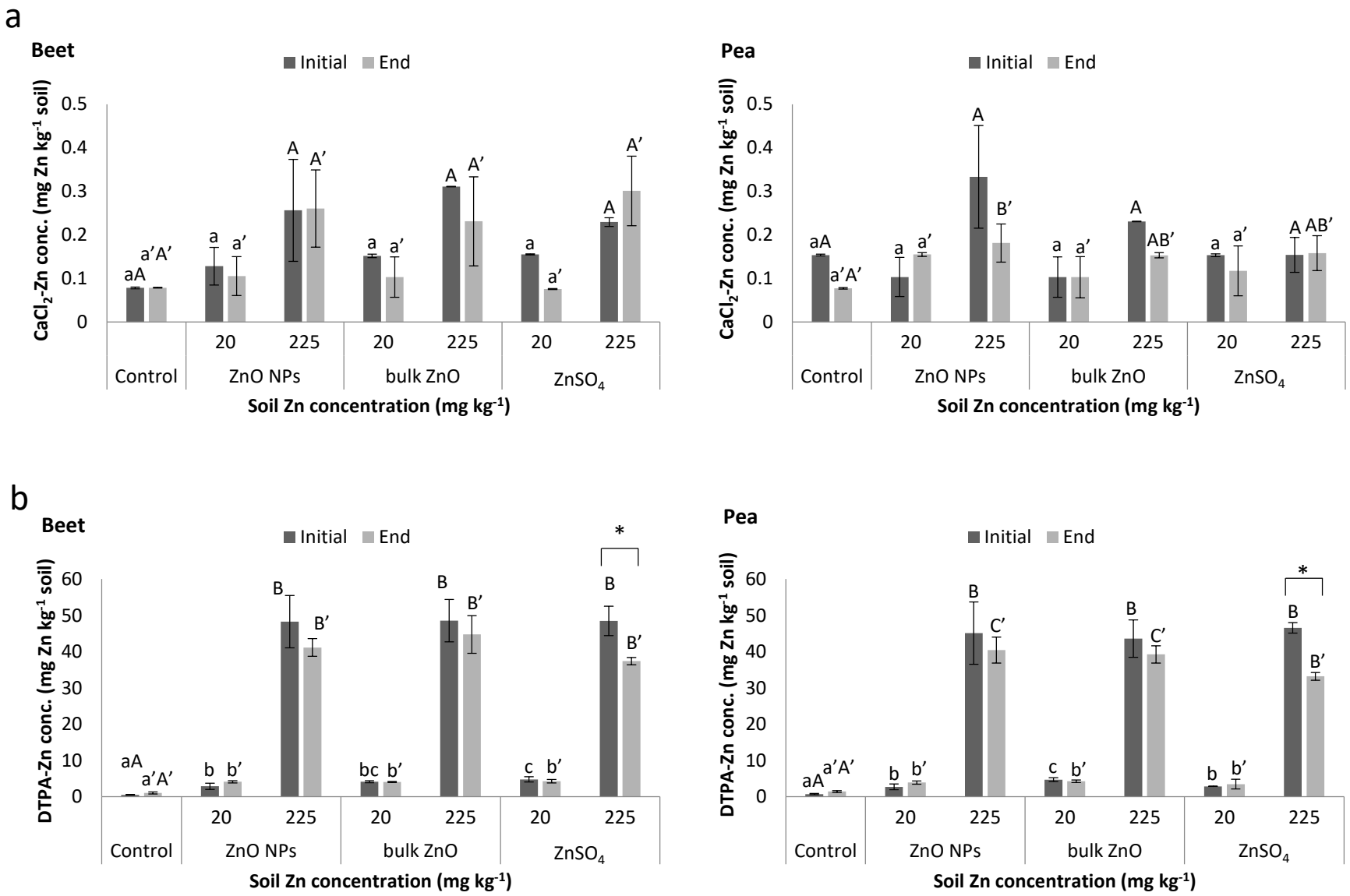

a
Beet
Initial
End
$CaCl_2$-Zn conc. (mg Zn kg^{-1} soil)
0.5
0.4
0.3
0.2
0.1
0
aA
a'A'
a
a'
A
A'
Control
20
225
ZnO NPs
bulk ZnO
$ZnSO_4$
Soil Zn concentration (mg kg^{-1})
Pea
AB'
B'
b
DTPA-Zn conc. (mg Zn kg^{-1} soil)
60
50
40
30
20
10
0
B
B'
bc
c
b'
C'
*

Figure 3

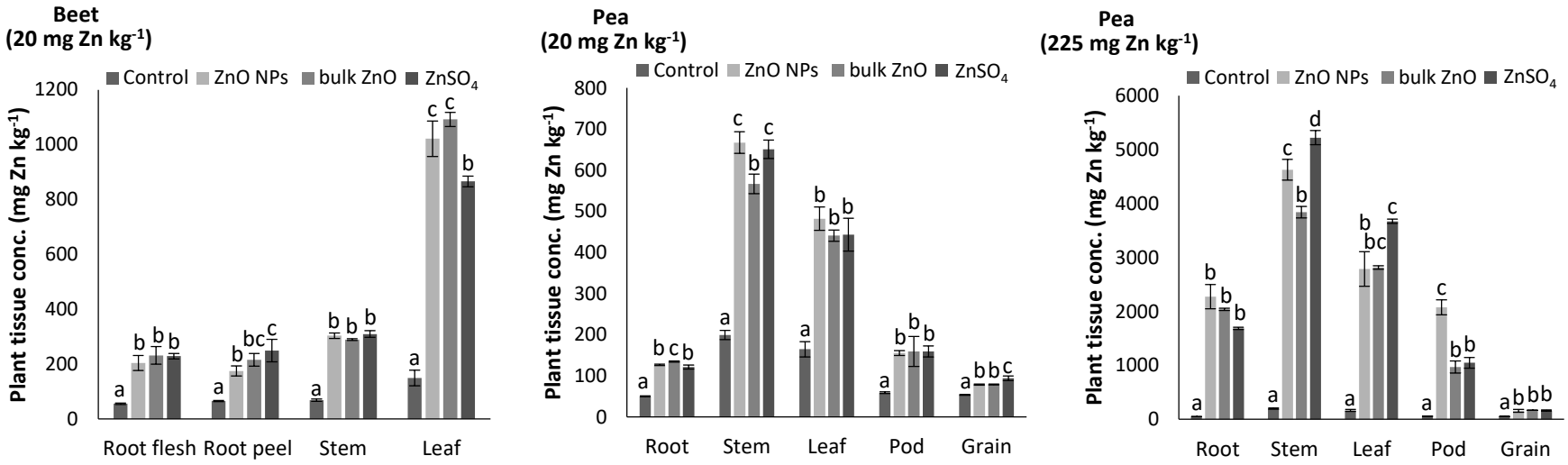

Beet
(20 mg Zn kg-1)
Control
ZnO NPs
bulk ZnO
ZnSO4
Plant tissue conc. (mg Zn kg-1)
0
200
400
600
800
1000
1200
Root flesh
Root peel
Stem
Leaf
Pea
(20 mg Zn kg-1)
Control
ZnO NPs
bulk ZnO
ZnSO4
Plant tissue conc. (mg Zn kg-1)
0
100
200
300
400
500
600
700
800
Root
Stem
Leaf
Pod
Grain
Pea
(225 mg Zn kg-1)
Control
ZnO NPs
bulk ZnO
ZnSO4
Plant tissue conc. (mg Zn kg-1)
0
1000
2000
3000
4000
5000
6000
Root
Stem
Leaf
Pod
Grain

Figure 4

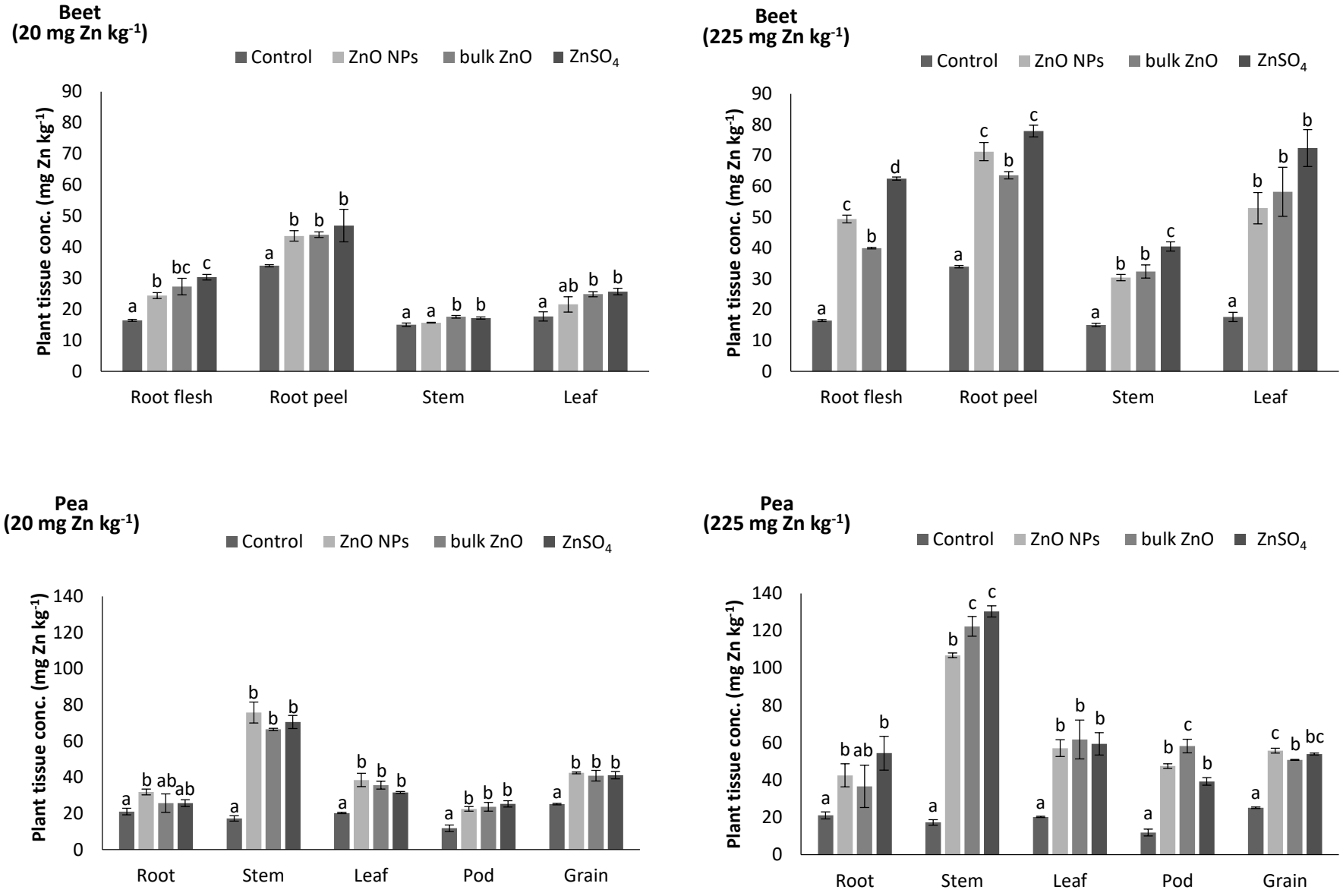

Beet
(20 mg Zn kg-1)
Control
ZnO NPs
bulk ZnO
ZnSO4
Plant tissue conc. (mg Zn kg-1)
Root flesh
Root peel
Stem
Leaf
Beet
(225 mg Zn kg-1)
Pea
(20 mg Zn kg-1)
Root
Stem
Leaf
Pod
Grain
Pea
(225 mg Zn kg-1)

Figure 5

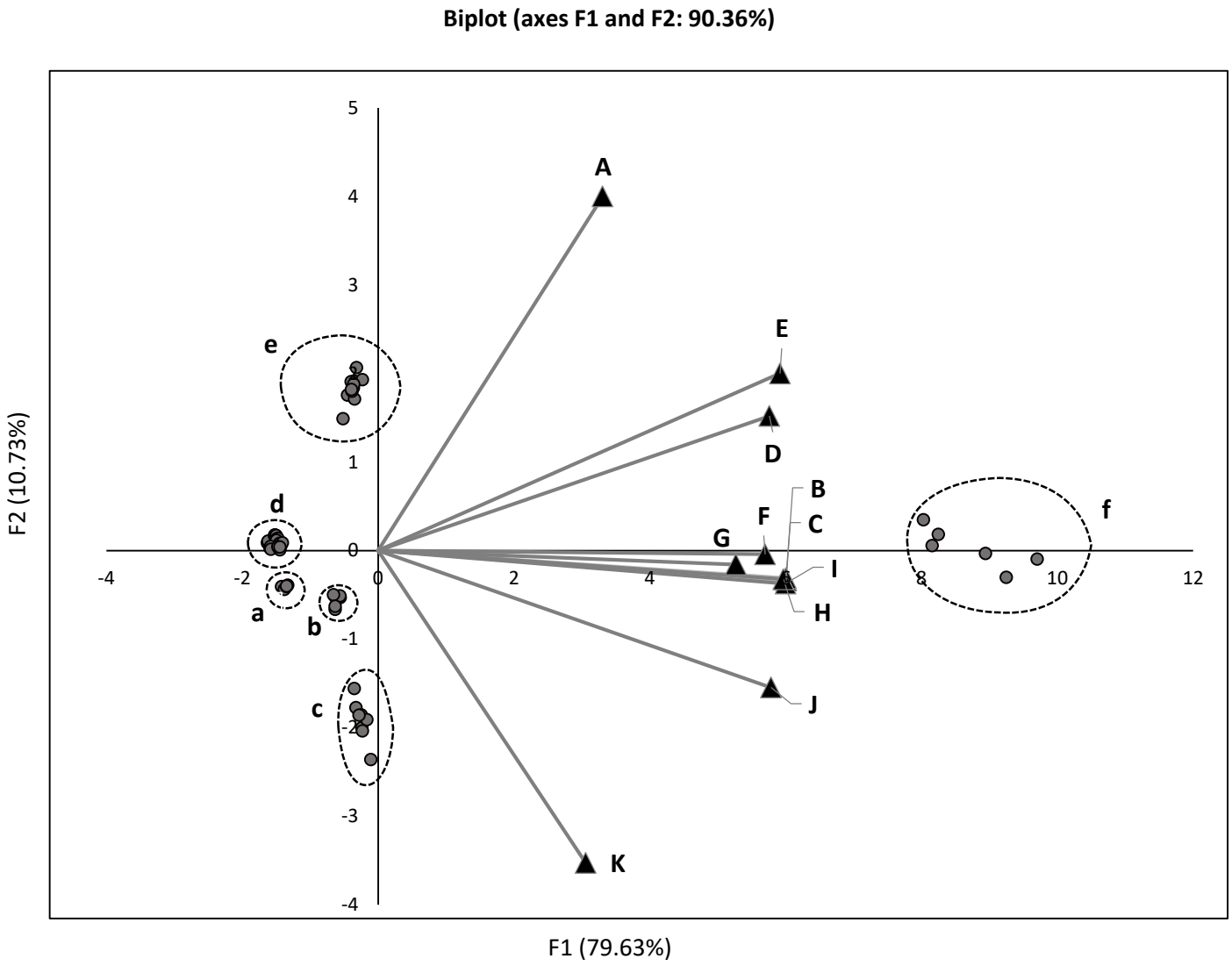
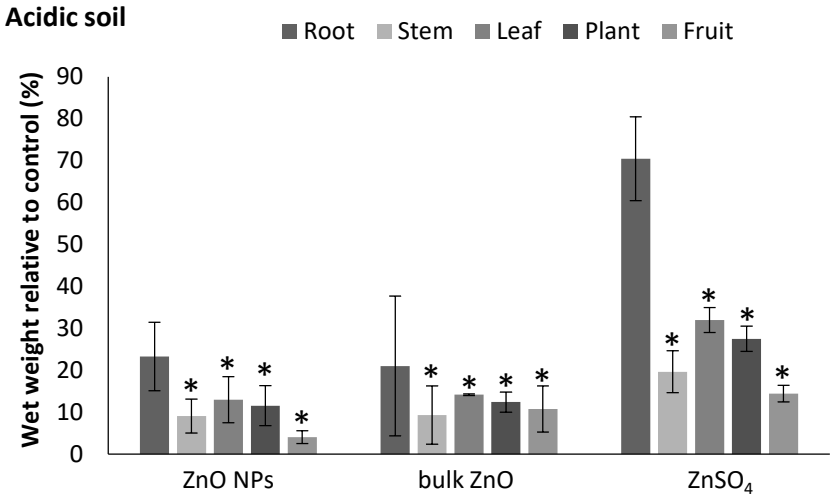
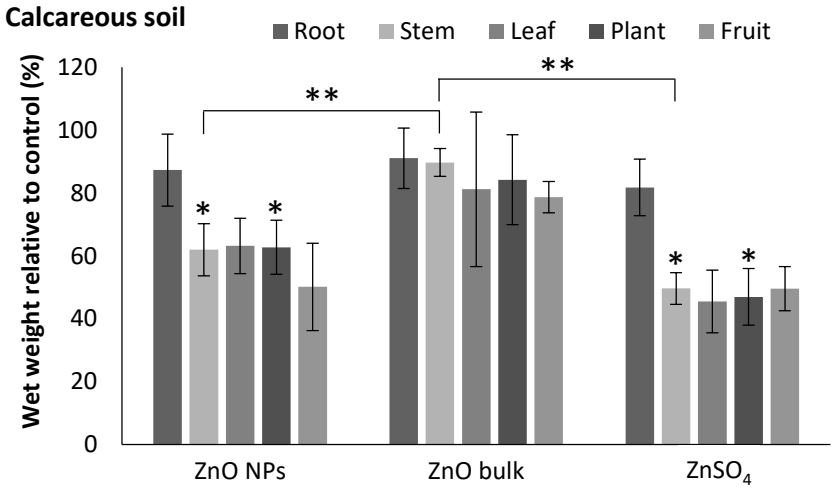

Biplot (axes F1 and F2: 90.36%)
F2 (10.73%)
F1 (79.63%)
A
B
C
D
E
F
G
H
I
J
K
a
b
c
d
e
f
5
4
3
2
1
0
-1
-2
-3
-4
-4
-2
0
2
4
8
10
12

Figure 6


Acidic soil
Root
Stem
Leaf
Plant
Fruit
Wet weight relative to control (%)
90
80
70
60
50
40
30
20
10
0
*
ZnO NPs
bulk ZnO
ZnSO4


Calcareous soil
Root
Stem
Leaf
Plant
Fruit
Wet weight relative to control (%)
120
100
80
60
40
20
0
**
*
ZnO NPs
ZnO bulk
ZnSO4