Soil and foliar zinc application to biofortify Broccoli (*Brassica oleracea* var. *italica* L.): effects on the zinc concentration and bioavailability

Abstract

Agronomic Zn biofortification of crops could help to alleviate dietary Zn deficiency, which is likely to affect more than one billion people worldwide. To evaluate the efficiency of agronomic Zn biofortification of broccoli, four application treatments were tested: no Zn application (control); soil application of 5 mg/kg $ZnSO_4.7H_2O$ (soil); two sprays (15 mL/pot each) of 0.25% (w/v) $ZnSO_4.7H_2O$ (foliar); and soil+foliar combination. Soil Zn application increased Zn-DTPA concentration by 3.7-times but did not affect plant growth or plant Zn concentration. Foliar Zn application increased stem+leaves and floret Zn concentration by 78 and 23 mg/kg Zn, respectively, with good bioavailability based on phytic acid concentration. Boiling decreased mineral concentration by 19%, but increased bioavailability by decreasing the phytic acid concentration. The entire broccoli could constitute a good nutritional source for animals and humans. An intake of 100 g boiled florets treated with the foliar treatment will cover about 36% of recommended dietary intake (RDI) of Zn, together with 30% of Ca, 94% of K, 32% of Mg, 6% of Na, 55% of P, 60% of S, 10% of Cu, 22% of Fe, 43% of Mn, and 35% of Se RDIs.

Keywords: Agronomic biofortification; soil zinc deficiency; zinc fertilizers; Brassicas; phytate

INTRODUCTION

Zinc (Zn) is an essential nutrient for crops, animals and humans. Its deficiency is associated with severe health complications including hindered physical growth and learning ability, neurological disorders, DNA damage and cancer development, causing death in extreme cases (Sanchez et al. 2009; Cakmak 2010). The Recommended Dietary Intake (RDI) is established at 15 mg/kg, however, ~20% of the world´s population is Zn deficient (WHO 2016). In Spain, about 56% of its population intake less than two thirds of this RDI (Sanchez et al. 2009). Drivers of Zn deficiency include: i) crops grown in soils with a low plant-availability of Zn; this includes a wide range of soil types worldwide, such as in the Mediterranean region, and limits crop yields and also Zn concentration in edible tissues (Cakmak et al. 2010); ii) the concentration of antinutrients in diets rich in plant food sources, mainly phytate which binds with Zn and other cations (e.g. Ca, Fe and Mg) and hinders their absorption in the human intestine (Gibson 2007); iii) a decrease in the amount and bioavailability of Zn during processing (Poblaciones and Rengel 2017a). Agronomic biofortification using foliar Zn application has been proved as an effective method for increasing the Zn concentration in the edible portions of several crops (Cakmak et al. 2010). Foliar application has also been shown to decrease phytate concentrations (Gomez-Coronado et al. 2016; Poblaciones and Rengel 2017a). Soil Zn application has lower effects on Zn and phytate concentrations than foliar applications but can improve yields on Zn-deficient soils (Cakmak et al. 2010; Gomez-Coronado et al. 2016).

Although several studies regarding agronomic biofortification have been developed in cereals and legumes, other crops as those belonging to *Brassica* genus have not received such attention despite being among the ten most economically important vegetables (Francisco et al. 2017). *Brassica* crops are an excellent dietary source of the main mineral and trace elements, vitamins and other organic nutrients (Moreno et al. 2006). Broccoli (*Brassica oleracea* var. *italica* L.) is the horticultural *Brassica* with the highest increase in surface in Spain. The Zn concentration of commercial broccoli florets has been reported to range from 21 mg/kg (Ogbede et al. 2015; Slosar et al. 2017) to 66 mg/kg (Kaluzewicz et al. 2016). There are limited studies on Zn biofortification in broccoli. Slosar et al. (2017) reported increases in floret Zn concentration of between 10 and 15% due to a foliar application of 375 and 750 g/ha Zn. White et al. (2018) established the critical shoot Zn concentration without loss of crop yield between 0.12 and 1.7 mg/g among different broccoli genotypes. The aim of this study was determine the effect of soil and foliar Zn biofortification on the yield and Zn concentration, including effects on Zn bioavailability, of processing, and other mineral element accumulation.

MATERIALS AND METHODS

The experiment was conducted in a naturally-lit greenhouse at School of Agronomy Engineering, Extremadura University, Badajoz, Spain (38°89′ N, 6°97′ W; 186 m above sea level). The greenhouse temperature during the experiment was 18 ± 6 ºC during the day and 12 ± 4 ºC during the night. A Xerofluvents sandy loam soil was collected from the area of Tierra de Barros region in Western Spain (38°88′ N, 7°04′ W). The soil was air-dried, sieved to 2 mm, and four subsamples were used to determine gravimetrically the texture (14.9% clay, 57.1% sand, 28.0% silt), soil pH, 6.5 ± 0.1, organic carbon 2.8 ± 0.1 g/kg, carbonates <1%, available phosphorus 15 mg/kg and potassium <15 mg/kg, nitrate nitrogen 1.3 mg/kg and ammonium nitrogen 2.7 mg/kg. This soil is considered as a Zn deficient soil according to Sims and Johnson (1991) with a plant-available Zn of 0.43 mg/kg soil determined according to Lindsay and Norvell (1978) by extraction with DTPA (diethylenetriamine pentaacetic acid) and measured by ICP-MS (Thermo Fisher Scientific iCAPQ, Bremen, Germany). Internal references and blanks were included every 24 samples.

The broccoli cultivar used was Green Top. Seeds were surface-sterilised by soaking in 80% v/v ethanol for 60 s, washed thoroughly with sterile water and sown in a seedbed containing substrate. After four weeks, plants were transplanted to 30-cm-high and 30-cm-wide free-draining pots containing 8.5 kg soil (one plant per pot). To ensure Zn was the only nutrient limiting growth, the following basal nutrients (in mg/kg) were added to soil as solutions: 90.2 KH_2PO_4; 139.9 K_2SO_4; 40.1 $MgSO_4.7H_2O$; 95.2 NH_4NO_3; 150.3 $CaCl_2.2H_2O$; 10.0 $MnSO_4.H_2O$; 2.0 $CuSO_4.5H_2O$; 0.5 $CoSO_4.7H_2O$; 0.2 $Na_2MoO_4.2H_2O$, 0.7 H_3BO_3. Soil Zn treatments (see below) consisted of spraying Zn sulphate solution to the soil surface. After application of basal nutrients and different soil Zn rates, the soil in each pot was thoroughly mixed. Extra application of 95.2 NH_4NO_3 mg/kg was applied each three weeks to avoid N deficiencies.

The experiment was arranged in completely randomized block design with four Zn treatments and four replicates. Treatments were: no Zn application (control); soil application of 5 mg/kg $ZnSO_4.7H_2O$ (soil); two sprays (15 mL/pot each) of 0.25%

(w/v) $ZnSO_4$-$7H_2O$ (foliar); and the combination of the soil and foliar applications (soil+foliar). Foliar treatments were applied once at the early beginning of flowering and the second two weeks after. Soil moisture content was maintained by watering plants every two days with deionised water. There was no incidence of pests or diseases during the study.

Plants were harvested at maturity 12 weeks after transplant, and carefully hand-washed with deionised water. Before harvest, four soil subsamples were took to determine plant-available Zn. Plant height and weight were measured before the floret was separated and weighed, together with floret height, higher diameter (D), and lower diameter (d). The floret was subdivided and subsampled for boiling, air dried at 60 ºC in a forced-air cabinet until constant weight, and weighed. The remaining subsample was boiled for 5 min in 400 mL of deionised water in Pyrex flasks. Total Zn concentration, together with Ca, K, Mg, Na, P, S, Cu, Fe, Mn and Se concentration, were measured in stem+leaves, florets and boiled florets. Accurately weighed powdered samples (each ~20 mg DW) were digested using a mix of nitric acid and hydrogen peroxide in a closed-vessel microwave system (Anton Paar Gmbh, Graz, Austria). Two blanks and two certified reference material (CRM: tomato leaf SRM 1573a NIST, Gaithersburg, MD, USA) were included every digestion run. The digested were determined by ICP-MS. The Zn-specific recovery from CRMs was 95% compared with certified CRM values.

Phytic acid (PA) was determined in all the samples as described by Reason et al. (2015) using a PA-total phosphorus assay kit (Megazyme, County Wicklow, Ireland)

and quantified by ultraviolet-visible spectroscopy at 655 nm. The molar ratio between PA and Ca, Fe, Mg and Zn was calculated.

Data were subjected to a one-way ANOVA for ‘Zn application’. Mineral concentrations were subjected to two-way ANOVA, including the ‘Broccoli part’, ‘Zn application’ as well as their interaction in the model. When significant differences were found, means were compared using Fisher’s protected least significant difference (LSD) test at $P < 0.05$. All analyses were performed using Statistix v. 8.10 for Windows (Analytical Software, Tallahassee, FL, USA).

RESULTS AND DISCUSSION

Soil Zn and plant growth. Only a slight decrease in DTPA-extractable soil Zn concentration was observed in control soils due to plant uptake. Soil application, in both, soil and soil+foliar significantly increased DTPA-extractable soil Zn concentration at plant harvest, up to 1.58 mg/kg (Table 1). Similar results were found by Poblaciones and Rengel (2017a) in Zn-deficient soils. Soil and foliar Zn application increased plant height, D and d significantly (Table 1), with a non-significant average increase in the floret weight of 8%. Slosar et al. (2017) reported floret yield increases of between 8.2 to 17.5% after foliar Zn application of 375 and 750 g/ha Zn applied as Zinkuran SC fertilizers. Abd El-All (2014) also found yield increase in broccoli when higher rates of foliar Zn fertilizers were applied three times during growth period again as Zinkuran SC fertilizers. White et al. (2018) did not find yield increases in different Brassicas after soil Zn application. This absence of significant yield increase in this current study could be due to: i) broccoli having a relative low sensitivity to soil Zn deficiency in the pot system used in this study, or ii) the Zn fertilizers were insufficient and/or that $ZnSO_4$ less efficient than other sources as Zn-EDTA (Zhao et al. 2018) or Zinkuran SC (Abd El-All 2014). These factors should be tested in field conditions where the size of the pot is not a limiting factor.

Nutritional composition in the different studied fractions. All the studied minerals, PA and PA:mineral ratios (except PA:Fe) varied widely depending on the analyzed broccoli part. Total Ca, Mg, Na, Mn and Zn concentrations were significantly higher in the stem+leaves than in the florets; total K, P, S, Cu, Fe and Se concentrations were significantly higher in the raw floret than in the stem+leaves (Table 2). Nutrient composition was largely similar to those found by Kaluzewicz et al. (2016) in ten broccoli cultivars, although with a slightly higher total K, P, Cu and Mg concentrations in the floret in the current study. Liu et al. (2018) found similar values for both, stem+leaves and floret in total Fe, Mg and Mn concentrations, higher in total Ca, K, Na (mainly in stem+leaves) and P concentrations, and lower in total Cu concentrations than the current study. These values could be directly related with the mineral concentrations in the soil used by Liu et al. (2018) which was rich in Ca, K, Mg, Na and Mn and from deficient to normal in P, S, Cu, Fe and Se.

The potential bioavailability of nutrients, measured by PA concentrations and the PA:mineral molar ratios, was greater in stem+leaves than florets, except for PA:Fe (Table 2). The PA:mineral molar ratios were less than their respective thresholds of 0.24 for PA:Ca (Morris and Ellis 1989); 10 for PA:Fe (Hallberg et al. 1989); and 0.2

for PA:Mg (Evans and Martin 1988). The PA:Zn molar ratios were less than 15 in stem+leaves (Gibson 2007) but higher in florets . These results highlight that the entire broccoli plant can constitute a good source of mineral nutrients for humans and livestock. In the study of Liu et al. (2018) , florets represents about 15% of total biomass, whereas, if stem and leaves were also consumed, then productivity of the broccoli crop would increase up to 83%.

Effect of Zn treatments on nutrient accumulation. Floret Zn concentration in the No-Zn treatment, 28.7 mg/kg Zn, was similar to that found by Slosar et al. (2017) (21 mg/kg Zn) but less than found by Kaluzewicz et al. (2016) (42 to 66 mg/kg Zn), due to a higher Zn-soil content. In stem+leaves, Zn concentration in the non-treated broccolis was only 7.8 mg/kg, much lower than that found by Liu et al. (2018). While soil application did not significantly alter Zn concentration in any of the studied parts, in foliar and soil+foliar treatments, the increases were larger in the stem+leaves than in the floret, 11.0 and 11.3-times more *vs* 1.67 and 1.88-times, respectively, compared to control treatments. Stem+leaves reached levels of 85.9 and 88.2 mg/kg Zn, respectively, almost 2-fold higher than their respective in the floret (Figure 1A). In all the cases, the levels are close to target breeding levels of HarvestPlus for legumes (Huett et al. 1997).

The PA concentration was significantly lower in stem+leaves than in the floret (2.1 *vs*. 7.7 g/kg) (Figure 1B). These values were lower than those found in cereals (Gomez-Coronado et al. 2016) or legumes (Poblaciones and Rengel, 2017a) similar for stem+leaves but higher in florets than those found by Ogbede et al. (2015) in cabbage and by Mohammed and Luka (2013) in green, red and Chinese cabbage, with contents between 2.2 to 3.1 g/kg.

The concentration of K was significantly greater in florets after foliar Zn treatments; Mn and P concentration were higher in florets in all Zn applications. The concentration of Se in florets was reduced after soil Zn application treatment but was unaffected by foliar Zn application (Table 3). Poblaciones and Rengel (2017b), found a positive effect of the combined application of foliar Se and Zn on the accumulation of Zn in field pea. Foliar Zn application reduced PA:Zn ratios (Table 3). The fact that foliar Zn application is not related with a decrease in the broccoli mineral composition or potential bioavailability is a key point. Broccoli is gaining consumers thanks to the good reputation that its mineral composition has and the implementation of a Zn biofortification program is not related to the loss of mineral quality.

Effect of processing. In broccoli, the most common processing method is boiling for about 5 min. A significant reduction of 36% in Zn concentration was found in florets because of boiling, and about 38% in PA as average in all Zn treatments (Figure 1). A small but significant reduction was found in K (22%), S (28%), Cu (27%), Mg (23%), Mn (12%), PA:Fe (27%) and PA:Mg (19%). This reduction was more drastic in Fe (33%), and PA:Ca (40%) (Table 2). Poblaciones and Rengel (2017a) found decreases of 12%, 16%, 15%, and 24% in grain Se, Ca, Mg, and Zn concentrations in field peas after frozen and boiling them and similar by Thavarajah et al. (2008) in lentils, with a longer cooking time and somewhat larger nutrient losses. Because of the decrease in the PA, the bioavailability of the broccoli florets has been increased.

According to the Recommended Dietary Intake (RDI) for males and females between 25 and 50 years published by FAO/WHO (2000) and the obtained results, an intake of 100 g of boiled broccoli treated foliarly with Zn will cover about: 32% of Ca, 91% of K, 32% of Mg, 6% of Na, 51% of P, 58% of S, 9% of Cu, 22% of Fe, 38% of Mn and about 35% of Se, with a good bioavailability according to Sandström (1989). According to the results, foliar was the best treatment from economically and biofortification points of view, along with an increase of total K, Mg, P, S and Fe of around 10% and of Cu and Mn around 20%. Regarding Zn, foliar applications would increase from 10% of the recommended 15 mg/day Zn up to 24%, reaching proportions of 57 and 59%, respectively, in the stem+leaves.

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Table 1. Broccoli yield characteristics and effect on plant-available soil Zn concentration under different agronomic Zn biofortification treatments (Soil Zn-DTPA, plant and floret heights and weights, higher (D) and lower (d) diameters means ± standard error of the mean; F values follow a one-way Analysis of Variance for Zn treatments).

Zn treatment	Soil Zn-DTPA (mg kg^{-1})	Plant weight (g)	Plant height (cm)	Floret height (cm)	Floret wheight (g)	D (cm)	d (cm)
F-value	6.23**	1.87	14.8**	2.77	3.50*	3.63*	3.51*
No-Zn	0.38 ± 0.04 b	314 ± 9.1	28.3 ± 0.5 b	16.6 ± 0.4	89.6 ± 5.5	8.7 ± 0.1 b	7.5 ± 0.2 b
Soil	1.58 ± 0.16 a	315 ± 19.1	31.0 ± 1.2 a	16.9 ± 0.9	96.3 ± 3.8	9.0 ± 0.4 ab	7.9 ± 0.3 ab
Foliar	0.45 ± 0.03 b	307 ± 3.6	31.3 ± 1.0 a	17.1 ± 0.6	96.4 ± 4.3	9.3 ± 0.3 a	8.0 ± 0.1 ab
Soil+Foliar	1.58 ± 0.19 a	292 ± 15.1	30.3 ± 0.6 a	16.1 ± 0.6	97.6 ± 3.2	9.6 ± 0.3 a	8.3 ± 0.2 a

Means in a column with different letters were significantly different (*P ≤ 0.05; **P ≤ 0.01) according to the Fisher's protected LSD test for the Zn treatment.

Table 2. Raw broccoli nutritional characteristics, phytic acid (PA) concentrations, and PA:mineral molar ratios under different agronomic Zn biofortification treatments (means ± standard error of the mean; F values follow a one-way Analysis of Variance for Zn treatments).

	Stem+leaves	**Floret**	**Boiled Floret**	**F value (Part)**
Total Ca (g/kg DW)	12.0 ± 0.6 a	2.4 ± 0.1 b	2.4 ± 0.1 b	306.47***
Total K (g/kg DW)	17.5 ± 0.5 c	24.0 ± 0.2 a	18.7 ± 0.3 b	114.79***
Total Mg (g/kg DW)	1.6 ± 0.1 a	1.3 ± 0.1 b	1.0 ± 0.1c	81.91***
Total Na (g/kg DW)	0.46 ± 0.03 a	0.37 ± 0.01 b	0.32 ± 0.01 b	13.63***
Total P (g/kg DW)	3.0 ± 0.1 b	4.5 ± 0.1 a	4.4 ± 0.1 a	178.23***
Total S (g/kg DW)	2.5 ± 0.1 c	6.7 ± 0.1 a	4.8 ± 0.1 b	436.95***
Total Cu (mg/kg DW)	0.8 ± 0.1 c	3.0 ± 0.2 a	2.2 ± 0.1 b	156.88***
Total Fe (mg/kg DW)	25 ± 4 b	40 ± 2 a	27 ± 1 b	10.76***
Total Mn (mg/kg DW)	19 ± 1 a	17 ± 1 b	15 ± 1 c	21.26***
Total Se (mg/kg DW)	0.13 ± 0.03 b	0.29 ± 0.06 a	0.22 ± 0.05 a	9.27***
Total Zn (mg/kgDW)	47.6 ± 10.9 a	39.3 ± 3.6 b	25.2 ± 2.6 c	31.05***
PA (g/kg DW)	2.21 ± 0.32 c	7.72 ± 0.22 a	4.82 ± 0.14 b	260.33***
PA:Ca	0.01 ± 0.01 c	0.20 ± 0.01 a	0.12 ± 0.01 b	217.43***
PA:Fe	0.85 ± 0.01	1.56 ± 0.01	1.14 ± 0.11	1.07
PA:Mg	0.05 ± 0.01 c	0.21 ± 0.01 a	0.17 ± 0.01 b	203.76***
PA:Zn	11.6 ± 2.41 b	21.9 ± 1.91 a	21.1 ± 2.22 b	37.61***

Means with different letters were significantly different (***P ≤ 0.001) according to the Fisher’s protected LSD test for the Zn treatment.

Table 3. Boiled broccoli nutritional characteristics, phytic acid (PA) concentrations, and PA:mineral molar ratios under different agronomic Zn biofortification treatments (means ± standard error of the mean; F values follow a one-way Analysis of Variance for Zn treatments)..

Zn treatment	Total K (g/kg DW)	Total P (g/kg DW)	Total Mn (mg/kg DW)	Total Se (mg/kg DW)	PA:Mg	PA:Zn
F value	3.91*	3.30*	9.74**	36.44***	3.65*	64.02***
No-Zn	19.3 ± 1.0 b	3.80 ± 0.21 b	15.9 ± 1.0 c	0.38 ± 0.01 a	0.14 ± 0.02 b	26.2 ± 2.4 a
Soil	19.6 ± 1.0 b	3.95 ± 0.19 ab	17.7 ± 0.9 b	0.05 ± 0.05 b	0.15 ± 0.02 b	26.3 ± 2.3 a
Foliar	20.7 ± 0.9 a	4.10 ± 0.24 a	19.4 ± 0.5 a	0.35 ± 0.01 a	0.14 ± 0.02 b	10.4 ± 2.3 b
Soil+Foliar	20.7 ± 0.9 a	3.90 ± 0.23 ab	16.5 ± 0.4 bc	0.35 ± 0.01 a	0.17 ± 0.02 a	11.2 ± 2.2 b

Means with different letters were significantly different (P ≤ 0.05) according to the Fisher’s protected LSD test for the Zn treatment.

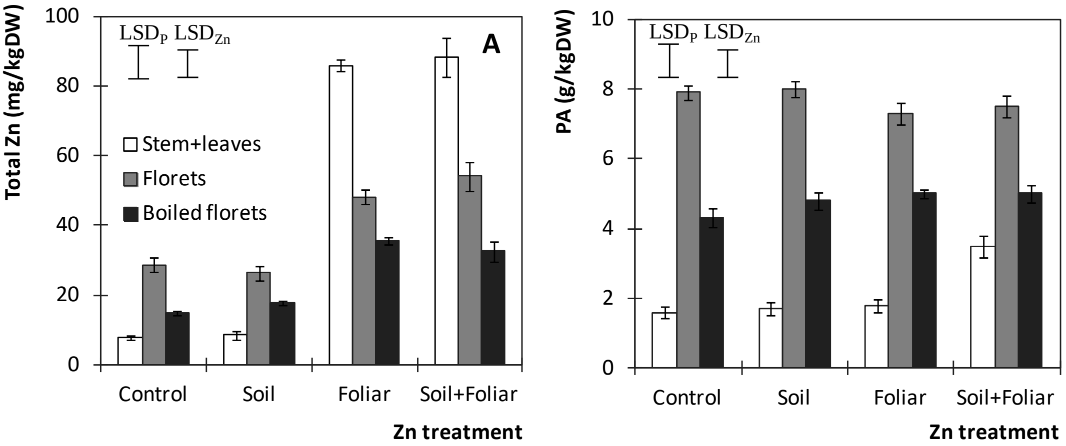

Figure 1. Total Zn (A) (mg/kg) and PA (B) concentration (g/kg) ± standard error of the mean. Vertical bars represent LSD ($P \leq 0.05$) for comparison: LSD$_P$, same broccoli part; LSD$_{Zn}$, same Zn treatment.