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Limits to the Biofortification of Leafy Brassicas with Zinc

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Received: 24 January 2018; Accepted: 22 February 2018; Published: 27 February 2018

Abstract: Many humans lack sufficient zinc (Zn) in their diet for their wellbeing and increasing Zn concentrations in edible produce (biofortification) can mitigate this. Recent efforts have focused on biofortifying staple crops. However, greater Zn concentrations can be achieved in leafy vegetables than in fruits, seeds, or tubers. Brassicas, such as cabbage and broccoli, are widely consumed and might provide an additional means to increase dietary Zn intake. Zinc concentrations in brassicas are limited primarily by Zn phytotoxicity. To assess the limits of Zn biofortification of brassicas, the Zn concentration in a peat:sand (*v*/*v* 75:25) medium was manipulated to examine the relationship between shoot Zn concentration and shoot dry weight (DW) and thereby determine the critical shoot Zn concentrations, defined as the shoot Zn concentration at which yield is reduced below 90%. The critical shoot Zn concentration was regarded as the commercial limit to Zn biofortification. Experiments were undertaken over six successive years. A linear relationship between Zn fertiliser application and shoot Zn concentration was observed at low application rates. Critical shoot Zn concentrations ranged from 0.074 to 1.201 mg Zn g^{-1} DW among cabbage genotypes studied in 2014, and between 0.117 and 1.666 mg Zn g^{-1} DW among broccoli genotypes studied in 2015–2017. It is concluded that if 5% of the dietary Zn intake of a population is currently delivered through brassicas, then the biofortification of brassicas from 0.057 to > 0.100 mg Zn g^{-1} DW through the application of Zn fertilisers could increase dietary Zn intake substantially.

Keywords: biofortification; *Brassica oleracea* L.; broccoli; cabbage; nutrition; toxicity; zinc

1. Introduction

It is estimated that over one-fifth of the world's population suffers from zinc (Zn) deficiency, which results in impaired development, ill health, and a reduction in gross domestic product [1–5]. One strategy to increase human dietary Zn intake is to increase Zn concentrations in edible produce. This strategy is termed biofortification and can be achieved through the use of Zn fertilisers on plant genotypes that have greater ability to acquire and accumulate Zn in their edible tissues [1,4–9]. Zinc might be applied to the soil as inorganic or organic fertilisers or to foliage as soluble salts [1,7,9–11]. Inorganic fertilisers are often preferred because of their consistent composition; foliar applications are most effective where the phytoavailability of Zn decreases rapidly when applied to the soil [1,5]. Recent biofortification efforts have focused largely on developing germplasm and agronomic strategies to increase Zn concentrations in staple crops including cereals, pulses, cassava and potatoes, and Zn

concentrations approaching 0.02–0.10 mg g^{-1} dry weight (DW), depending upon the crop, have been achieved [1,6,9,12–21]. However, greater Zn concentrations can be achieved in leafy vegetables than in fruits, seeds or tubers because Zn transport in the phloem limits Zn accumulation in the latter tissues [13]. Zinc concentrations in leafy vegetables appear to be limited primarily by Zn phytotoxicity, suggesting that concentrations of 0.10–0.70 mg Zn g^{-1} DW shoot might be achieved without loss of yield [13]. Thus, Zn biofortification of leafy vegetables might also provide a means to increase Zn intake by human populations.

Leafy vegetables are a significant source of micronutrients for human populations, especially those with low incomes or with a vegetarian diet [3,4]. Brassicaceous vegetables, such as cabbage (*Brassica oleracea* var. *capitata*) and broccoli (*B. oleracea* L. var. *italica*), are among the most commonly consumed and economically important vegetables in the world [22,23]. The health benefits of brassicaceous vegetables are not only associated with their mineral composition but also their vitamin content and the presence of other organic compounds, particularly glucosinolates [22,24,25]. Although leafy vegetables currently contribute proportionally less Zn to human diets than animal products or cereals [3,4], their greater potential for Zn biofortification could be exploited to increase Zn intake and improve human health.

A large variation in shoot Zn concentration has been reported among the genotypes of *B. oleracea* [26–32]. For example, shoot Zn concentration among 36 cabbage genotypes grown together in a field in Himachal Pradesh, India, ranged from 0.002 to 0.005 mg Zn g^{-1} fresh weight [29]; significant differences in leaf Zn concentrations were observed among three cabbage genotypes grown together in the field in Pennsylvania, USA [26]; floret Zn concentrations of 10 broccoli genotypes grown together in Poznań, Poland, ranged from 0.042 to 0.066 mg Zn g^{-1} DW [30]; the average shoot Zn concentration of 22 kale (*B. oleracea* var. *acephala*) genotypes grown together in the field in New Hampshire, USA, over two years ranged from 0.033 to 0.060 mg Zn g^{-1} DW [27]; and leaf Zn concentrations of 6 kale genotypes grown in the field in KwaZulu-Natal Province, South Africa, ranged from 0.025 to 0.032 mg Zn g^{-1} DW [33]. However, although the heritability of shoot Zn concentration in *B. oleracea* is significant and several chromosomal quantitative trait loci (QTL) affecting shoot Zn concentration have been identified in this species, the heritability is often low and the QTL identified depends on the growth conditions [28,29,34]. The aim of the experiments reported here was, therefore, to determine the limit to Zn biofortification—using a Zn fertiliser—of two leafy brassicas, cabbage and broccoli, which contribute significantly to human diets worldwide. The Zn concentration in a peat:sand (v/v 75:25) potting medium was manipulated using zinc nitrate in order to examine the relationship between shoot Zn concentration and shoot dry biomass. This was to determine the critical shoot Zn concentration—defined as the shoot Zn concentration at which the yield was reduced below 90% [35]—of different genotypes. This value was regarded as the commercial limit to Zn biofortification. The manipulation of shoot Zn concentration through Zn applications to the substrate was necessary because the Zn concentrations of shoot tissues are difficult to determine unambiguously following the application of foliar Zn fertilisers. The Zn concentration in the substrate was manipulated using an inorganic Zn fertiliser rather than an organic amendment to avoid any potential effects of other components of organic amendments.

2. Materials and Methods

2.1. Plant Material

Five cabbage (*Brassica oleracea* L. var. *capitata*) genotypes were selected for study on the basis of their potentially large leaf zinc (Zn) concentrations based on data presented by [28]. The genotypes Bison, Cape Horn and Red Drumhead were grown in 2012, 2013 and 2014. The genotype Elisa was grown only in 2012. The genotype Tundra was grown only in 2013 and 2014. Seeds of Bison, Cape Horn, Red Drumhead and Tundra were obtained from Kings Seeds (Colchester, UK) and seeds of Elisa were obtained from Thompson & Morgan (Ipswich, UK). Four broccoli (*Brassica oleracea* L. var. *italica*) genotypes, Belstar, Chevalier, Marathon and Waltham 29, were studied in 2015, 2016 and 2017.

These genotypes were selected on the basis of their potentially contrasting leaf Zn concentrations based on data presented by [28]. Seeds of Belstar, Chevalier, Marathon and Waltham 29 were obtained from Van Meuwen (Spalding, UK), Kings Seeds Direct (Colchester, UK) and Unwins (Huntingdon, UK).

2.2. Growth Conditions

Seeds of cabbage were germinated in square Petri dishes (length × width × depth = 100 mm × 100 mm × 18 mm; Camlab, Cambridge, UK) on blue germination paper (Anchor Paper Company, St Paul, MN, USA) moistened with 10 mL deionised water. One week after placing the seeds in the Petri dishes, three to ten germinated seedlings of a genotype were transplanted to each pot containing 1 litre of the potting medium described in the next paragraph. Seedlings were thinned to a density of one seedling per pot two weeks after transplanting and grown for a further four weeks before harvesting. Ten seeds of broccoli were sown directly into pots containing 1 litre of potting medium. Broccoli seedlings were thinned to a density of one seedling per pot three weeks after sowing and grown for a further six weeks before harvesting. Thus, cabbage was harvested six weeks after transplanting pre-germinated seedlings to pots and broccoli was harvested nine weeks after sowing seeds into pots. Both cabbage and broccoli plants were at the true leaf/rosette stage of development, immediately prior to heading.

Plants were grown in an unheated glasshouse at The James Hutton Institute, Dundee, UK (latitude 56.4566° N, longitude 3.0708° W) in a potting medium, henceforth described as a "substrate", with Zn concentrations ranging from sufficient to phytotoxic. The standard substrate was made in bulk each year and comprised 75% peat (Sinclairs Professional Peat, Sinclair Pro, Ellesmere Port, UK) and 25% sand mixed with 0.225 g L^{-1} single superphosphate, 0.4 g L^{-1} ammonium nitrate, 0.75 g L^{-1} potassium nitrate, 2.25 g L^{-1} ground limestone, 2.25 g L^{-1} Magnesian limestone and 0.51 g L^{-1} of a trace element mixture containing 0.16% boron, 0.79% copper, 11.82% iron, 1.97% manganese, and 0.04% molybdenum by weight. The density of the substrate was 625 g L^{-1} when dry and 822 g L^{-1} when watered to holding capacity. The standard substrate contained 9.4 mg Zn L^{-1} and <0.1 mg L^{-1} water-extractable Zn (n = 6) prior to any further additions.

The standard substrate was thoroughly mixed and sieved before zinc nitrate was added to achieve the specified Zn concentrations. Zinc nitrate was employed rather than zinc sulphate to avoid any potential effect of increasing sulphate bioavailability on Zn accumulation. Six treatments were established in all experiments. In 2012, the treatments were the additions of 0, 0.075, 0.15, 1.5, 150 or 3000 mg Zn L^{-1} substrate. In 2013, the treatments were the additions of 0, 0.15, 150, 500, 1000 or 1500 mg Zn L^{-1} substrate. In 2014, 2015, 2016 and 2017 the treatments were the additions of 0, 1.5, 15, 150, 300 or 450 mg Zn L^{-1} substrate. The substrate was watered to holding capacity immediately before the experiments. When watered to holding capacity the pH of the solution in the substrate was ca. 6.8. Three replicate pots of each genotype x treatment were established in 2012 and 2013. Ten replicate pots of each genotype x treatment were established in 2014, 2015, 2016 and 2017. Pots were arranged in a blocked design with one replicate of each genotype x treatment combination in a random location within each block.

Surviving plants were harvested on 12 September 2012, 27 June 2013, 22 July 2014, 29 July 2015, 5 October 2016 and 25 July 2017. The accumulated temperature x time during plant growth in the glasshouse was 606.7 °C day in 2012, 525.3 °C day in 2013, 638.7 °C day in 2014, 820.1 °C day in 2015, 929.4 °C day in 2016 and 908.9 °C day in 2017. The accumulated solar radiation in the glasshouse during plant growth was 524.0 MJ m^{-2} in 2012, 702.5 MJ m^{-2} in 2013, 716.3 MJ m^{-2} in 2014, 1046.4 MJ m^{-2} in 2015, 702.4 MJ m^{-2} in 2016 and 1011.7 MJ m^{-2} in 2017.

2.3. Plant Analysis

Shoot fresh weight (FW) was determined at harvest and shoot dry weight (DW) was determined following drying to a constant weight in an oven at 70 °C. Zinc concentrations were determined following acid digestion of dried shoot material using inductively coupled plasma mass spectrometry (ICP-MS) as described by White et al. [36]. Accurately weighed subsamples (c. 50 mg DW) were

digested in closed vessels using a microwave digester (MARS Xpress, CEM Microwave Technology, Buckingham, UK). Samples were first digested with 10 mL concentrated nitric acid (HNO_3) before 3 mL of 30% hydrogen peroxide (H_2O_2) was added to each vessel and digestion completed. Digested samples were diluted with milliQ (sterile, 18.2 MΩ cm) water before Zn concentrations were determined using ICP-MS (PerkinElmer ELAN, DRCe, Monza, Italy). Blank digestions were performed to determine background Zn concentrations and a tomato leaf standard (Reference 1573a; National Institute of Standards and Technology, NIST, Gaithersburg, MD, USA) was used as an analytical control.

2.4. Statistics

Data are expressed as the mean ± standard error of the mean (SE) for n observations. The relationships between shoot biomass (W) and the zinc concentration (Zn) in the substrate or shoot tissue were fitted to a sigmoidal function: W = a/(1 + EXP(b*((Zn) − c))) + d, where the minimum biomass equals d, the maximum biomass equals a + d, c is the (Zn) at the point of inflection and the slope of the relationship at the point of inflection is given by −ab/4.

3. Results

The relationships between substrate fertiliser Zn concentration, which is less than the actual substrate Zn concentration but is henceforth termed 'substrate Zn concentration' for brevity, and shoot fresh weight (FW), shoot dry weight (DW) and shoot Zn concentration of cabbage seedlings were studied in experiments performed in 2012, 2013 and 2014 (Table S1). The experiments performed in 2012 and 2013 narrowed the range of substrate Zn concentrations required to determine the 'critical' substrate and shoot Zn concentrations at which shoot DW was 90% of its maximal value [13]. The experiment performed in 2012 indicated that neither shoot FW nor shoot DW of cabbage genotypes was greatly reduced at substrate concentrations up to 150 mg L^{-1} and that shoot Zn concentration increased as the substrate Zn concentration was increased (Table S1). The genotype Elisa did not grow well in the substrate employed in these experiments and was replaced by Tundra in subsequent experiments. The experiment performed in 2013 indicated that the sensitivity of shoot FW and shoot DW to increasing substrate Zn concentration followed the sequence Red Drumhead > Bison > Cape Horn > Tundra (Table S1). The critical substrate Zn concentration at which shoot DW was 90% of its maximal value was < 0.15 mg L^{-1} for Red Drumhead, between 0.15 and 150 mg L^{-1} for Bison and between 150 and 500 mg L^{-1} for Cape Horn and Tundra. Shoot Zn concentration increased linearly with increasing substrate Zn concentration with gradients between 7.2 and 13.6 mg Zn kg^{-1} DW/mg Zn L^{-1} (Bison = 9.9, Cape Horn = 8.4, Red Drumhead = 13.6, Tundra = 7.2). The critical shoot Zn concentrations were <0.18 mg g^{-1} DW for Red Drumhead, between 0.05 and 1.65 mg g^{-1} DW for Bison, between 1.36 and 4.27 mg g^{-1} DW for Cape Horn and between 1.05 and 3.73 mg g^{-1} DW for Tundra. Experiments in both 2012 and 2013 indicated considerable variation in the responses of individual plants of all genotypes grown under identical conditions to substrate Zn concentration, and that a large number of replicates would be required to obtain a more precise estimate of the critical substrate and shoot Zn concentrations for shoot DW accumulation.

In the experiments performed on cabbage in 2014, increasing substrate Zn concentration above about 100–150 mg L^{-1} reduced shoot DW of all cabbage genotypes studied (Figure 1A–D). However, estimates of critical substrate Zn concentrations were relatively imprecise. For Bison, the relationship between substrate Zn concentration and shoot DW could be fitted with a sigmoidal function, indicating a critical substrate Zn concentration of 96 mg fertiliser Zn g^{-1} DW substrate (Figure 1A, Table 1). Shoot DW of the Cape Horn genotype increased greatly with the addition of 0.15 mg L^{-1} Zn to the substrate, suggesting that the Zn in the substrate itself was insufficient to support maximal growth of this genotype in this experiment. The relationship between substrate Zn concentration above 0.15 mg L^{-1} and shoot DW could, however, be fitted by a sigmoidal function indicating a critical substrate Zn concentration of 100 mg L^{-1} (Figure 1B, Table 1). The relationship between substrate Zn concentration and shoot DW for Red Drumhead could not be fitted by a sigmoidal function but

indicated a critical substrate Zn concentration of 5 mg L^{-1} (Figure 1C, Table 1). The genotype Tundra was relatively insensitive to the addition of <450 mg Zn L^{-1} and was estimated to have a critical substrate Zn concentration of 260 mg L^{-1} (Figure 1D, Table 1). The ranking of genotypes and estimates of critical values were generally consistent with data obtained in 2013.

Increasing the substrate Zn concentration increased shoot Zn concentration in all cabbage genotypes (Figure 1E–H). The relationship between substrate Zn concentration and shoot Zn concentration was linear in the genotypes Bison and Tundra, with a gradient of 5.80 and 4.62 L kg^{-1} DW, respectively, but asymptotic in Cape Horn and Red Drumhead, reaching a maximum of 1.70 and 1.91 mg Zn g^{-1} DW, respectively (Figure 1I–L, Table 2). From the experiments performed in 2014, the critical shoot Zn concentrations of Bison, Cape Horn, Red Drumhead and Tundra were estimated to be 0.79, 0.80, 0.074 and 1.20 mg Zn g^{-1} DW, respectively (Figure 1I–L, Table 1). The ranking of genotypes and the estimates of critical values were generally consistent with data obtained in 2013.

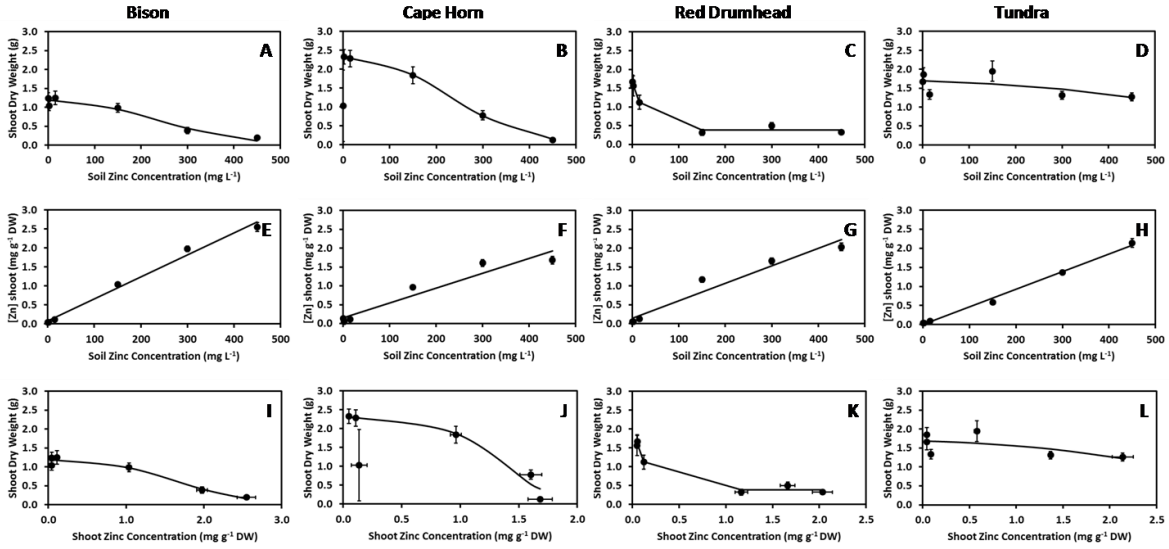

Figure 1. Response of cabbage genotypes to the addition of zinc (Zn) fertiliser to the substrate. *Top row*: Relationship between the addition of Zn fertiliser to substrate (substrate Zn concentration, (Zn)) and shoot dry weight (DW) of (**A**) Bison, (**B**) Cape Horn, (**C**) Red Drumhead and (**D**) Tundra genotypes grown in 2014. *Middle row*: Relationship between substrate Zn concentration and shoot Zn concentration of (**E**) Bison, (**F**) Cape Horn, (**G**) Red Drumhead and (**H**) Tundra genotypes grown in 2014. Parameters for linear regression lines are given in Table 2. *Bottom row*: Relationship between shoot Zn concentration ((Zn)) and shoot dry weight of (**I**) Bison, (**J**) Cape Horn, (**K**) Red Drumhead and (**L**) Tundra genotypes. Regression lines are fitted to the equation DW = a/(1 + EXP(b*((Zn) − c))) + d, where the minimum biomass equals d, the maximum biomass equals a + d, c is the substrate (**A**–**D**) or shoot (**I**–**L**) Zn concentration at the point of inflection and the slope of the relationship at the point of inflection is given by −ab/4. For all lines d = 0 and a is the maximum shoot biomass. Parameters a, b and c were: (**A**) a = 1.26, b = 0.0114, c = 248 for Bison, (**B**) a = 2.41, b = 0.0130, c = 241 for Cape Horn, (**C**) not fitted for Red Drumhead, (**D**) a = 1.77, b = 0.0049, c = 633 for Tundra, (**I**) a = 1.21, b = 2.19, c = 1.68 for Bison, (**J**) a = 2.31, b = 4.18, c = 1.31 for Cape Horn, (**K**) not fitted for Red Drumhead, (**L**) a = 1.77, b = 1.05, c = 2.91 for Tundra. Data are shown as the mean and standard error of the mean (Table S1).

The relationships between substrate Zn concentration and shoot FW, shoot DW and shoot Zn concentration of broccoli seedlings were studied in experiments performed in 2015, 2016 and 2017 (Table S1; Figures 2–4). In all years there was considerable variation in the responses of individual plants of all genotypes grown under identical conditions to substrate Zn concentration (Table S1) and estimates of critical substrate and shoot Zn concentrations were relatively imprecise (Figures 2 and 4). Shoot DW was reduced by increasing substrate Zn concentration in all years in all genotypes (Figure 2).

However, the estimated critical substrate Zn concentration for shoot DW differed between years and, apparently, among genotypes (Table 1). The critical substrate Zn concentration was greater in 2017, ranging from 271 to 408 mg Zn L^{-1} among genotypes, than in 2016, ranging from 43 to 139 mg Zn L^{-1} among genotypes, and 2015, ranging from 107 to 121 mg Zn L^{-1} among genotypes (Table 1). In 2015, the critical substrate Zn concentration was similar for all genotypes but in both 2016 and 2017, the shoot DW of Belstar appeared less sensitive to substrate Zn concentration than the other cultivars, whereas the shoot DW of Waltham 29 was among the most sensitive genotypes to substrate Zn concentration (Table 1).

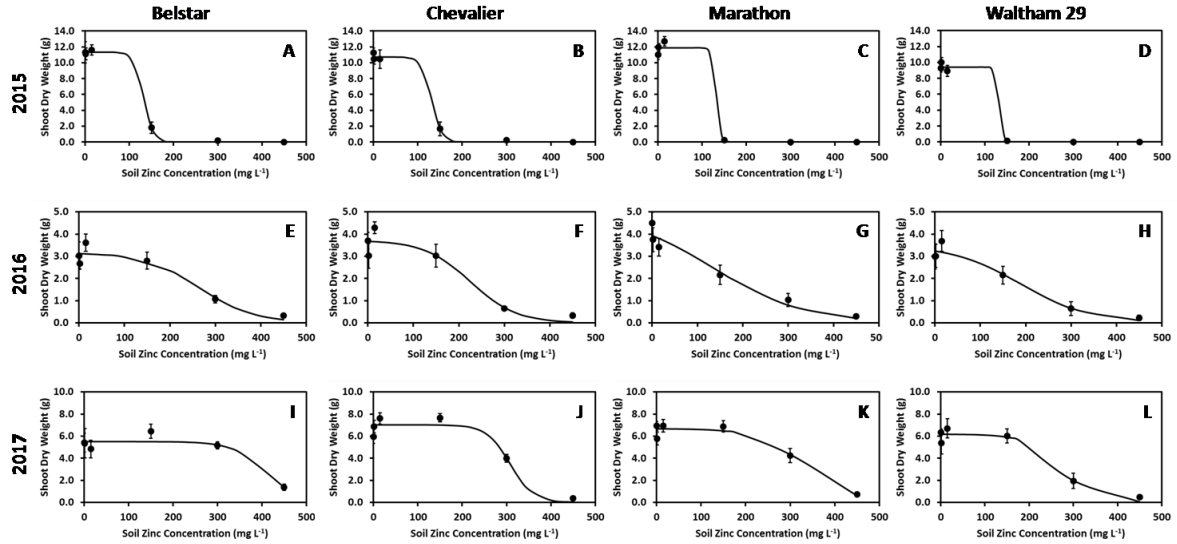

Figure 2. Relationship between substrate zinc concentration (Zn) and shoot dry weight (DW) of (**A**,**E**,**I**) Belstar, (**B**,**F**,**J**) Chevalier, (**C**,**G**,**K**) Marathon and (**D**,**H**,**L**) Waltham 29 broccoli genotypes grown in 2015 (**top row**), 2016 (**middle row**) and 2017 (**bottom row**). Regression lines are fitted to the equation DW = a/(1 + EXP(b*((Zn) − c))) + d, where the minimum biomass equals d, the maximum biomass equals a + d, c is the substrate Zn concentration at the point of inflection and the slope of the relationship at the point of inflection is given by −ab/4. For all lines d = 0 and a is the maximum shoot biomass. Parameters a, b and c were: (**A**) a = 11.3, b = 0.0893, c = 131 for Belstar in 2015, (**B**) a = 10.7, b = 0.0862, c = 130 for Chevalier in 2015, (**C**) a = 11.9, b = 0.2041, c = 131 for Marathon in 2015, (**D**) a = 9.40, b = 0.2181, c = 131 for Waltham 29 in 2015, (**E**) a = 3.16, b = 0.0164, c = 265 for Belstar in 2016, (**F**) a = 3.72, b = 0.0197, c = 225 for Chevalier in 2016, (**G**) a = 4.95, b = 0.01, c = 135 for Marathon in 2016, (**H**) a = 3.51, b = 0.0132, c = 189 for Waltham 29 in 2016, (**I**) a = 5.51, b = 0.0264, c = 408 for Belstar in 2017, (**J**) a = 7.03, b = 0.0380, c = 308 for Chevalier in 2017, (**K**) a = 6.68, b = 0.0193, c = 332 for Marathon in 2017, (**L**) a = 6.18, b = 0.0255, c = 271 for Waltham 29 in 2017. Data are shown as the mean and standard error of the mean (Table S1).

Shoot Zn concentration generally increased linearly with increasing substrate Zn concentration, although this relationship had an exponential tendency in Chevalier, Marathon and Waltham 29 in 2015 and tended towards an asymptotic maximum in Marathon in 2016 (Figure 3). The shoots of plants grown in 2015 had greater shoot Zn concentrations than those grown in 2016, which had greater shoot Zn concentrations than those grown in 2017 when grown in the same substrates (Table S1, Figure 3). Genotypes differed in their relationship between shoot Zn concentration and substrate Zn concentration (Figure 3). In general, the shoot Zn concentration in Belstar increased the least and the shoot Zn concentration in Waltham 29 the most, with increasing substrate Zn concentration across the three years of the study (Figure 3).

Table 1. Maximum shoot biomass and critical substrate zinc (Zn) and shoot Zn concentrations at which shoot dry biomass was reduced below 90%, estimated from the relationships shown in Figures 1 and 2 derived from the data expressed on a dry weight (DW) basis presented in Supplementary Table S1.

Year	Genotypes	Maximum Shoot Biomass		Critical Zn Concentration	
		Substrate Zn Regression (g DW)	Shoot Zn Regression (g DW)	Substrate (mg L^{-1})	Shoot (mg g^{-1} DW)
Cabbage					
2014	Bison	1.07	1.06	96	0.789
2014	Cape Horn	2.08	2.06	100	0.802
2014	Tundra	1.52	1.52	260	1.201
2014	Red Drumhead	1.48	1.45	5	0.074
Broccoli					
2015	Belstar	11.33	11.33	107	0.434
2015	Chevalier	10.72	10.72	105	0.499
2015	Marathon	11.87	11.87	121	0.514
2015	Waltham 29	9.40	9.64	121	0.117
2016	Belstar	3.12	3.12	139	1.018
2016	Chevalier	3.68	3.68	120	0.901
2016	Marathon	3.54	3.55	43	0.277
2016	Waltham 29	2.91	2.91	68	0.406
2017	Belstar	5.51	5.51	408	1.666
2017	Chevalier	7.03	7.03	308	1..424
2017	Marathon	6.68	6.70	332	1.527
2017	Waltham 29	6.18	6.24	271	1.195

Table 2. Gradients and intercepts for linear regressions between shoot zinc (Zn) concentrations and substrate Zn concentrations of four cabbage genotypes studied in 2014 and four broccoli genotypes studied in 2015, 2016 and 2017.

Year	Gradient (mg Zn kg^{-1} DW/mg Zn L^{-1}), Intercept (mg Zn kg^{-1} DW)			
	Bison	**Cape Horn**	**Tundra**	**Red Drumhead**
2014	5.802, 73.64	5.209, 85.92 (A)	4.620, 4.706	5.641, 83.76 (A)
	Belstar	**Chevalier**	**Marathon**	**Waltham 29**
2015	7.126, 70.16	5.170, 5.644 (E)	5.015, 12.83 (E)	6.853, 36.36 (E)
2016	5.654, 74.84	5.714, 80.16	5.894, 88.42	6.350, 12.32
2017	3.997, 49.46	4.837, 8.768	4.680, 7.523	4.452, 11.53

Data are regressions based on six substrate Zn concentrations, except for the cabbage genotypes Cape Horn and Red Drumhead in 2014 and the broccoli genotypes Chevalier, Marathon and Waltham 29 in 2015, which are regressions based on five substrate Zn concentrations $\leq$300 mg Zn L^{-1}. (A) Asymptotic, and (E) exponential regressions shown in Figures 1 and 3.

Shoot DW decreased with increasing shoot Zn concentration in all years in all genotypes (Figure 4). However, the estimated critical shoot Zn concentration for shoot DW accumulation differed between years and, apparently, among genotypes (Table 1). In general, shoot DW of Belstar appeared less sensitive to shoot Zn concentration than the other cultivars, particularly in 2016 and 2017, whilst Waltham 29 was among the most sensitive genotypes to shoot Zn concentration (Table 1). In 2015, the critical shoot Zn concentrations ranged between 0.117 mg g^{-1} DW for Waltham 29 and 0.514 mg g^{-1} DW for Marathon. In 2016, the critical shoot Zn concentrations ranged between 0.277 mg g^{-1} DW for Marathon and 1.018 mg g^{-1} DW for Belstar. In 2017, the critical shoot Zn concentrations ranged between 1.195 mg g^{-1} DW for Waltham 29 and 1.666 mg g^{-1} DW for Belstar.

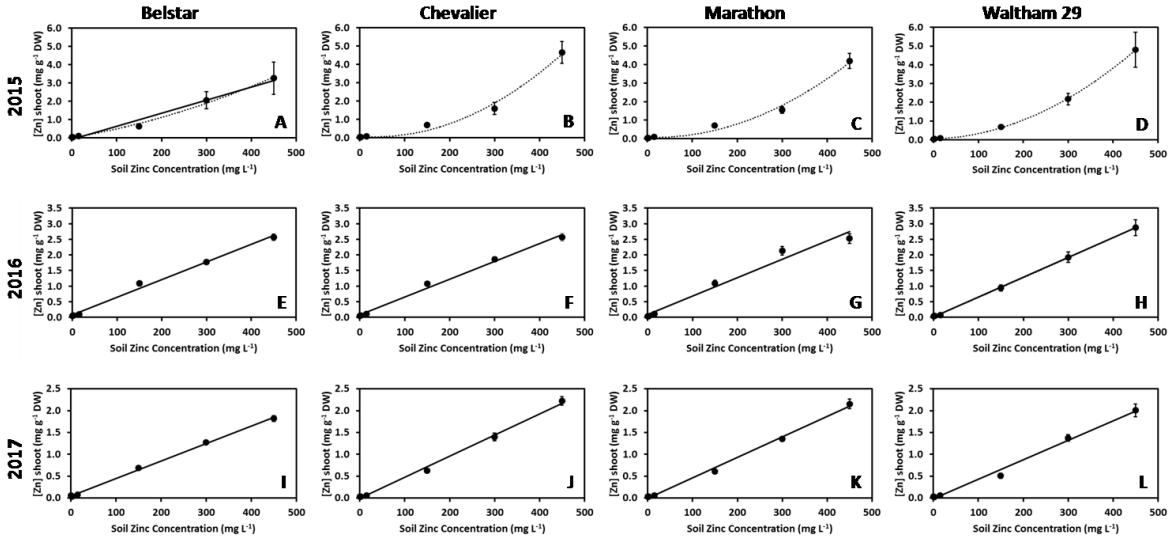

Figure 3. Relationship between substrate zinc (Zn) concentration and shoot Zn concentration of (**A**,**E**,**I**) Belstar, (**B**,**F**,**J**) Chevalier, (**C**,**G**,**K**) Marathon and (**D**,**H**,**L**) Waltham 29 broccoli genotypes grown in 2015 (**top row**), 2016 (**middle row**) and 2017 (**bottom row**). Parameters for linear regression lines are given in Table 2. Data are shown as the mean and standard error of the mean (Table S1).

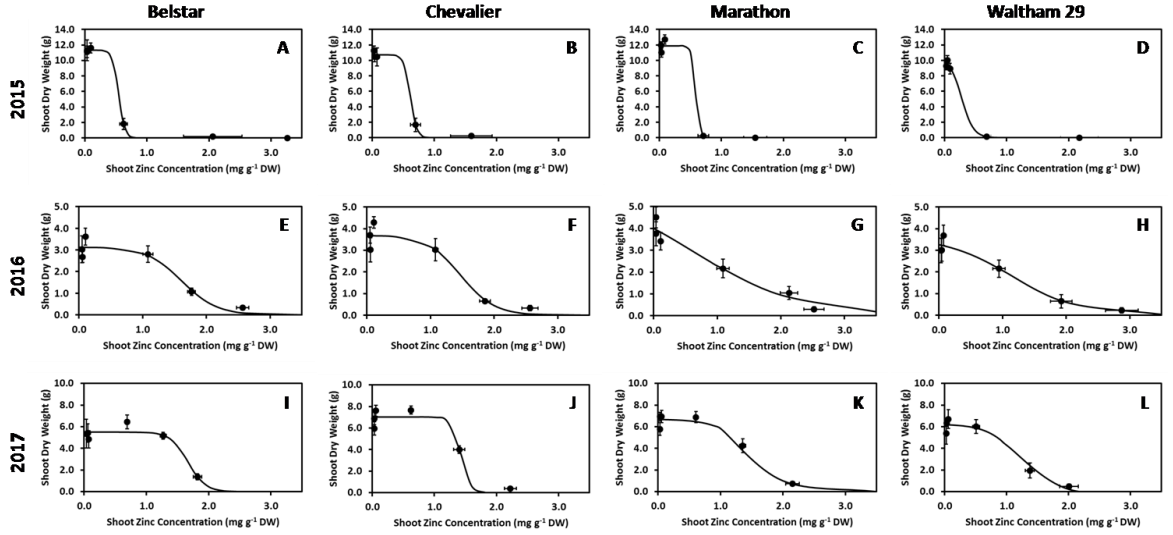

Figure 4. Relationship between shoot zinc concentration (Zn) and shoot dry weight (DW) of (**A**,**E**,**I**) Belstar, (**B**,**F**,**J**) Chevalier, (**C**,**G**,**K**) Marathon and (**D**,**H**,**L**) Waltham 29 broccoli genotypes grown in 2015 (**top row**), 2016 (**middle row**) and 2017 (**bottom row**). Regression lines are fitted to the equation $DW = a/(1 + EXP(b*((Zn) - c))) + d$, where the minimum biomass equals d, the maximum biomass equals a + d, c is the substrate Zn concentration at the point of inflection and the slope of the relationship at the point of inflection is given by $-ab/4$. For all lines d = 0 and a is the maximum shoot biomass. Parameters a, b and c were: (**A**) a = 11.3, b = 20.3, c = 0.542 for Belstar in 2015, (**B**) a = 10.7, b = 19.5, c = 0.612 for Chevalier in 2015, (**C**) a = 11.9, b = 30.8, c = 0.585 for Marathon in 2015, (**D**) a = 10.4, b = 10.5, c = 0.272 for Waltham 29 in 2015, (**E**) a = 3.13, b = 3.63, c = 1.61 for Belstar in 2016, (**F**) a = 3.69, b = 3.75, c = 1.47 for Chevalier in 2016, (**G**) a = 5.97, b = 1.19, c = 0.596 for Marathon in 2016, (**H**) a = 3.64, b = 1.89, c = 1.14 for Waltham 29 in 2016, (**I**) a = 5.51, b = 7.17, c = 1.66 for Belstar in 2017, (**J**) a = 7.02, b = 13.1, c = 1.42 for Chevalier in 2017, (**K**) a = 6.70, b = 3.66, c = 1.53 for Marathon in 2017, (**L**) a = 6.24, b = 4.05, c = 1.20 for Waltham 29 in 2017. Data are shown as the mean and standard error of the mean (Table S1).

There was no relationship between shoot DW of broccoli genotypes and either the critical substrate Zn concentration or the critical shoot Zn concentration (data not shown). However, although estimates of both the critical substrate Zn concentration and the critical shoot Zn concentration differed among experiments (Table 1), there was a linear relationship between the critical shoot Zn concentration and the critical substrate Zn concentration both among genotypes in 2016 and 2017 and among experiments performed in different years (Figure 5A). Data for cabbage genotypes obtained in 2014 also exhibited a similar relationship (Figure 5A). Thus, plants that are better able to tolerate the accumulation of Zn in their shoots can survive and grow in substrates with larger substrate Zn concentrations. There was also a negative relationship between the critical substrate Zn concentration and the initial linear rate of change of Zn uptake with increasing substrate Zn concentration in broccoli (Figure 5B), which might be explained because greater shoot Zn accumulation at any given substrate Zn concentration will result in a lower critical substrate Zn concentration should all other factors remain constant. However, there was also a negative relationship between the critical shoot Zn concentration and the initial linear rate of change of Zn uptake with increasing substrate Zn concentration in broccoli (Figure 5C). This observation might suggest that tolerance to increasing shoot Zn concentration is promoted by (unknown) substrate factors that change with increasing substrate Zn concentrations. Similar negative relationships between both the critical substrate and shoot Zn concentrations and the initial linear rate of change of Zn uptake with increasing substrate Zn concentration were also observed in cabbage (Figure 5B,C).

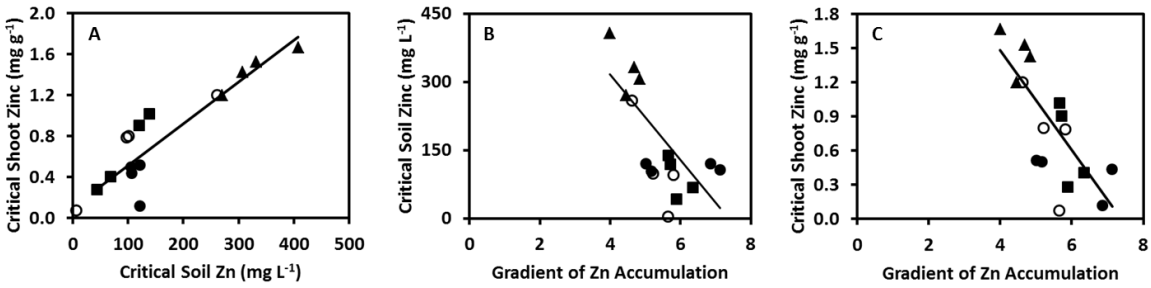

Figure 5. Relationship between (**A**) critical shoot zinc (Zn) concentrations and critical substrate Zn concentrations, (**B**) critical substrate Zn concentrations and the linear rates of change of Zn uptake with increasing substrate Zn concentration and (**C**) critical shoot Zn concentrations and the linear rate of change of Zn uptake with increasing substrate Zn concentration among four genotypes of broccoli studied in 2015 (closed circles), 2016 (squares) and 2017 (triangles), and four genotypes of cabbage studied in 2014 (open circles). The regression line in panels A ($y = 4.09x + 101$ mg kg^{-1}/mg L^{-1}, $R^2 = 0.840$, $n = 12$) and B ($y = -0.438x + 3.232$, $R^2 = 0.640$, $n = 12$) and C ($y = -93.96x + 693.2$, $R^2 = 0.585$, $n = 12$) are for broccoli only. Data are presented in Tables 1 and 2.

4. Discussion

Increasing the substrate Zn concentration above a critical value reduced the shoot DW of all cabbage and broccoli genotypes studied (Table S1, Figures 1 and 2). The critical substrate fertiliser Zn concentration, which is less than the total available Zn in the substrate but approximates the Zn readily available to the plant in the substrate, varied between years and differed among the genotypes, ranging from 5 to 260 mg Zn L^{-1} among cabbage genotypes studied in 2014 and between 43 and 408 mg Zn L^{-1} for the broccoli genotypes studied in 2015–2017 (Table 1). These values are much lower than statutory maximum annual Zn loading rates to soils in Europe and elsewhere [37].

The phytoavailability of soil Zn is affected by many factors [38]. These are often soil-specific. A major determinant of Zn phytoavailability in soils is pH but soil organic matter content, mineral and clay composition, porosity and moisture content are also influential. In addition, although brassicas are non-mycorrhizal, interactions with soil biota can also affect Zn phytoavailability in soils. It is, therefore, unwise to extrapolate from the critical substrate fertiliser Zn concentrations obtained in the

experiments reported here (Table 1) directly to farmers' fields. It is also noteworthy that in soils where the phytoavailability of Zn applied as fertiliser decreases rapidly, the Zn biofortification of crops is generally achieved by foliar application of Zn fertilisers [1,5,9,11,13,15,20,37].

A variety of relationships between substrate Zn concentration and shoot Zn concentration were observed (Figures 1 and 3). A linear relationship between substrate Zn concentration and shoot Zn concentration was often observed but an asymptotic relationship toward a maximum shoot Zn concentration was observed for cabbage genotypes Cape Horn and Red Drumhead in 2014 and an exponential relationship was observed for broccoli genotypes Chevalier, Marathon and Waltham 29 in 2015. Previous studies of cabbage suggest a linear relationship between soil Zn concentration and shoot Zn concentration [39,40]. It is possible that the asymptotic relationship towards a maximum shoot Zn concentration was a consequence of measuring shoot Zn concentrations of only plants that survived the presence of a given substrate Zn concentration, which might have restricted Zn accumulation.

Differences in the gradients of the relationship between substrate Zn concentration and shoot Zn concentration were observed among both cabbage and broccoli genotypes (Table 1). Among the cabbage genotypes, shoot Zn concentrations were greatest in Bison and least in Tundra (Table S1, Figure 1), which is consistent with the observations of Broadley et al. [28] when ample phosphorus was supplied. Among the broccoli genotypes, Waltham 29 generally had the greatest shoot Zn concentration and Belstar the smallest shoot Zn concentration when Zn fertiliser was applied, although this order was reversed when no Zn fertiliser was applied (Table S1, Figure 3). These data reinforce previous studies reporting significant differences in shoot Zn concentrations among both cabbage [26,29] and broccoli genotypes [30,31]. Shoot Zn concentrations determined at the same substrate Zn concentration differed between years for all broccoli genotypes (Table S1, Figure 3). A similar observation was made by [27], who reported that there were significant differences in the shoot Zn concentrations of kale and collard genotypes grown in different years in the field. The differences reported here might be related to the season in which the plants were grown and, therefore, the glasshouse temperature, incident photosynthetically active radiation, day length or another uncontrolled environmental factor. However, no obvious relationships between accumulated temperature (°C day) or solar radiation were observed for the relationships between substrate Zn concentration, shoot Zn concentration and shoot DW.

Critical shoot Zn concentrations ranged from 0.074 to 1.201 mg Zn g^{-1} DW among the cabbage genotypes studied in 2014 and between 0.117 and 1.666 mg Zn g^{-1} DW among the broccoli genotypes studied in 2015–2017 (Table 1). The smallest values are within the range (0.1–0.3 mg Zn g^{-1} DW) that is commonly quoted for critical shoot Zn concentrations of plants [35,41] and the largest values exceed current estimates of the Zn biofortification potential of leafy vegetables (0.7 mg Zn g^{-1} DW; [13]). The cabbage genotypes studied appeared to have very different critical shoot Zn concentrations, ranging from 0.074 mg Zn g^{-1} DW for Red Drumhead to 1.201 mg Zn g^{-1} DW for Tundra (Table 1; Figure 1). These data are consistent with previous studies indicating that the critical shoot Zn concentration of cabbage approximates 0.05–0.40 mg Zn g^{-1} DW [35,40,42,43] and that cabbage genotypes differ in their critical shoot Zn concentration [44]. Differences in critical shoot Zn concentration appeared to be less pronounced among the broccoli genotypes studied, although Waltham 29 appeared more sensitive and Belstar less sensitive than the other genotypes to increasing shoot Zn concentration (Table 1; Figure 4). Estimates of critical shoot Zn concentrations for broccoli genotypes differed between years (Table 1, Figure 4). This might be attributed to differences in the growth environment in different years. Variation in critical shoot Zn concentration is observed among studies of other brassicaceous species, such as *Brassica napus* L. (compare [45–49]) and *Brassica juncea* (L.) Czern [50–52] that might be related to either the genotypes studied or the experimental conditions.

5. Conclusions

Recent efforts to biofortify edible crops with Zn have focused primarily on staple crops, such as cereals, pulses, cassava and potatoes, and maximum Zn concentrations of 0.02–0.10 mg g^{-1} DW,

depending upon the crop, have been achieved without loss of yield [1,6,9,12–21]. The critical shoot Zn concentrations in cabbage and broccoli reported here generally exceed these values (Table 1). This supports the general hypothesis that greater Zn concentrations can be achieved in leafy vegetables than in seeds, roots or tubers [13]. Furthermore, since leafy brassicas, unlike the seeds of legumes and cereals, do not contain large concentrations of phytic acid, the Zn in cabbage and broccoli should be readily bioavailable to humans [8]. This observation could have implications for increasing dietary Zn intake and the alleviation of Zn deficiencies in human populations. If it is assumed that brassicas constitute 5% of the Zn in current diets [3,4], increasing their shoot Zn concentrations from 0.057 mg Zn g^{-1} DW—the average value obtained without the addition of Zn fertilisers in the six experiments reported here (Table S1)—to 0.10–0.30 mg Zn g^{-1} DW through the application of Zn fertilisers to appropriate genotypes could increase dietary Zn intake by 3.8–21.3% without loss of crop yield. This has the potential to raise the Zn status and general health of human populations without any necessity for people to change their diets. However, there might be socioeconomic constraints to developing a strategy to alleviate Zn deficiencies in human populations through Zn biofortification of leafy vegetables because (1) the application of Zn fertilisers for Zn biofortification is an additional production cost and (2) appropriate infrastructure is required to distribute Zn fertilisers to produce Zn biofortified crops and to distribute Zn biofortified crops to populations that lack sufficient Zn in their diets [1].

Supplementary Materials: The following are available online at http://www.mdpi.com/2077-0472/8/3/32/s1, Table S1: The number of replicate plants surviving and the mean fresh weight (FW), dry weight (DW) and zinc (Zn) concentrations of the shoots of five cabbage (*Brassica oleracea* L. var. *capitata*) genotypes (Bison, Cape Horn, Elisa, Red Drumhead and Tundra) grown in 2012, 2013 and 2014 in a standard substrate into which no Zn, 0.075 mg Zn L^{-1} substrate, 0.15 mg Zn L^{-1} substrate, 1.5 mg Zn L^{-1} substrate, 15 mg Zn L^{-1} substrate, 150 mg Zn L^{-1} substrate, 300 mg Zn L^{-1} substrate or 450 mg Zn L^{-1} substrate, 500 mg Zn L^{-1} substrate, 1000 mg Zn L^{-1} substrate, 1500 mg Zn L^{-1} substrate or 3000 mg Zn L^{-1} substrate was incorporated depending on the year of the study, and four broccoli (*Brassica oleracea* L. var. *italica*) genotypes (Belstar, Chevalier, Marathon and Waltham 29) grown in 2015, 2016 and 2017 in a standard substrate into which no Zn, 1.5 mg Zn L^{-1} substrate, 15 mg Zn L^{-1} substrate, 150 mg Zn L^{-1} substrate, 300 mg Zn L^{-1} substrate or 450 mg Zn L^{-1} substrate was incorporated. Data for FW, DW and shoot Zn concentrations are expressed as the mean ± standard error of the mean (SE) for *n* surviving replicate plants.

Acknowledgments: This work was supported by the Rural and Environment Science and Analytical Services (RESAS) Division of the Scottish Government, by an EU Marie Curie Intra-European Fellowship (Grant #623305) to Paula Pongrac, and by a Rank Prize Funds Vacation Studentship to Claire Sneddon.

Author Contributions: All authors conceived and designed the experiments; C.C.S., J.A.T. and G.W. performed the experiments; C.C.S. and P.J.W. analysed the data; P.J.W. drafted the manuscript; all authors commented on the work.

Conflicts of Interest: The authors declare no conflicts of interest.

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