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Zinc from foliar-applied nanoparticle fertiliser is translocated to wheat grain: A ^{65}Zn radiolabelled translocation study comparing conventional and novel foliar fertilisers

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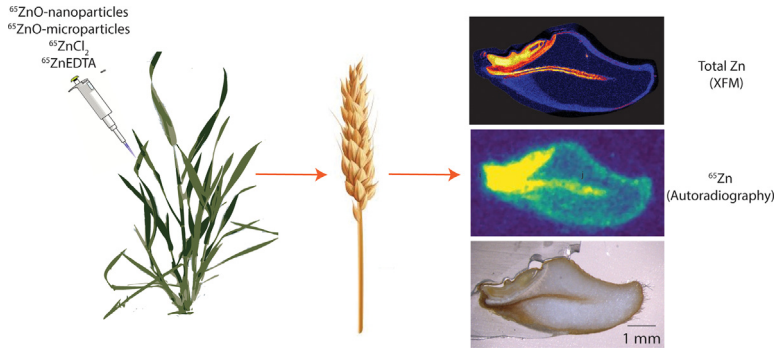
HIGHLIGHTS

- Zinc from nanoparticle foliar fertiliser is translocated to wheat grain
- The distribution of zinc in grain is the same for foliar and soil Zn applications
- Radiolabelled zinc fertilisers were used to trace zinc in wheat plants
- Conventional foliar Zn fertilisers are more efficient than novel nano formulations
- Lower zinc application rates are significantly more efficient

GRAPHICAL ABSTRACT



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ABSTRACT

Foliar zinc (Zn) fertilisers can be used to supplement or replace soil applications of Zn in situations where soil properties may decrease the plant bioavailability of Zn. However, conventional foliar Zn formulations such as zinc sulfate can cause leaf damage due to the rapid release of high amounts of Zn^{2+} into leaf tissue which can be locally phytotoxic. Zinc oxide nanoparticles (ZnO-NPs) offer an alternative approach by providing a more sustained release of Zn into leaf tissue, and potentially avoiding the need for multiple applications. We compared the efficacy of ZnO-NPs and microparticles (ZnO-MPs) to that of conventional formulations ($ZnCl_2$ and ZnEDTA) in wheat. This is the first study to use ^{65}Zn radiolabelled formulations and gamma spectrometry to determine the translocation of Zn to the grains and subsequent efficiency of foliar-applied ZnO-NP fertilisers. We found that ZnEDTA was the most efficient fertiliser in terms of the proportion of applied Zn translocated to wheat grain. We also investigated the effect of Zn application rate on fertiliser efficiency. For all forms of Zn, when plants were treated with Zn at 750 mg/L or 75 mg/L, there were no significant differences in the concentration of applied Zn translocated to the grain. This suggests that current Zn application rates could be decreased while still maintaining the nutritional quality of grain. Finally, using photo-stimulated luminescence (PSL) autoradiography and synchrotron-based X-ray fluorescence microscopy (XFM) we showed that the grain distribution of foliar-applied Zn mirrors that of Zn derived from root uptake.

1. Introduction

Zinc (Zn) deficiency is one of the most widespread micronutrient deficiencies in humans, with up to 30% of populations in developing

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countries at risk of inadequate Zn intake (Wessells and Brown, 2012). Inadequate dietary Zn can have a range of deleterious health effects including growth retardation, impaired brain function and compromised immune function (Prasad, 1985). As a staple food, cereals represent one of the most common sources of dietary Zn. Therefore, biofortification (i.e. increasing the nutrient content of food crops during plant growth) of cereal crops with Zn can be undertaken to ensure adequate levels of Zn are present in plant parts consumed by humans. One strategy to achieve this is agronomic biofortification where Zn is applied as fertiliser. Zinc fertilisers are applied to cultivated soils that are inherently low in Zn, and/or soils with properties that act to decrease Zn bioavailability (e.g. calcareous alkaline soils where Zn is absorbed and precipitated by $CaCO_3$ and Ca-hydroxides (Papadopoulos and Rowell, 1989). The latter issue can be overcome by using foliar Zn applications to supplement soil applications. The most commonly used Zn foliar sprays are soluble Zn solutions (e.g. Zn sulfate; $ZnSO_4$ and Zn chloride; $ZnCl_2$) and chelated Zn formulations (ZnEDTA) (Montalvo et al., 2016); however, both have drawbacks. Leaf damage is very common when soluble Zn is applied at typical agronomic rates (≥1000 mg Zn/L) (Cakmak and Kutman, 2018). Zinc-EDTA is significantly more costly than $ZnSO_4$ (Datta et al., 2015), and there are environmental concerns regarding the environmental persistence of EDTA and its ability to mobilise metals in aquatic environments (Oviedo and Rodríguez, 2003). To overcome these issues, nanoparticulate forms of Zn have been suggested as an alternative delivery method (Kopittke et al., 2019). Over recent years, Zn oxide nanoparticles (ZnO-NPs) have garnered much research interest. Zinc-oxide NPs may provide a more sustained release of Zn^{2+}, with Zn^{2+} being the main form of Zn most likely absorbed by the leaf (Li et al., 2018), attributable to ZnO-NPs being more soluble than bulk ZnO (65 μg/100 g, (Wang et al., 2016)), but less soluble than $ZnSO_4$ (63 g/100 g (Bury, 1924)). And, compared to other nanoparticle formulations, ZnO-NPs rapidly dissolve when they enter soil (Wang et al., 2013); therefore the potential environmental risks of ZnO-NPs are unlikely to differ to that of soluble Zn.

The efficacy of foliar-applied ZnO-NPs has been investigated in a variety of plant species. In foxtail millet (*Setaria italica* L.), foliar ZnO-NPs did not increase grain yield compared to untreated plants, but did increase the seed oil content (Kolenčík et al., 2019) – where millet oil can provide nutritional benefit in the form of polyunsaturated fatty acids (Shi et al., 2015). However, a positive control (e.g. conventional formulation such as $ZnSO_4$) was not included in the study. When ZnO-NPs were applied to pinto bean (*Phaseolus vulgaris*), Zn seed content increased significantly more than that for $ZnSO_4$ and chelated Zn treatments (Mahdieh et al., 2018). Similarly in habanero pepper plants (*Capsicum chinense* Jacq.), García-López et al. (2019) found that ZnO-NPs applied at 2000 mg/L negatively affected plant growth but improved fruit quality parameters (e.g. fruit firmness). For wheat – one of the most targeted crops for Zn biofortification – there is a paucity of data on the effect of foliar-applied ZnO-NPs on grain Zn. Most wheat studies have focused on short-term Zn uptake (e.g. 24 h Zn exposure and 14 d plant growth) (Read et al., 2019), or soil applications of ZnO-NPs (Gupta and Sharma, 2019; Milani et al., 2012; Stewart et al., 2015), where soil application has not been shown to be more beneficial than other forms of Zn (Kopittke et al., 2019). To our knowledge, only one study (Zhang et al., 2018) has investigated the effect of foliar ZnO-NPs on Zn grain content. In a wheat field trial carried out within the Loess Plateau on a typical Zn deficient soil [diethylenetriaminepentaacetic (DTPA) – Zn < 0.05 mg/kg], the authors found that foliar application of ZnO-NPs at 2000 mg/L did not increase grain yield or grain quality, but did increase grain Zn concentration significantly more than $ZnSO_4$ applied at 7000 mg/L.

One of the challenges of evaluating ZnO-NP foliar fertilisers, is that absorbed Zn cannot be distinguished from Zn that has been taken up from the roots, or from the seed. Therefore, most translocation and mobility studies have relied on the use of control plants to estimate background concentrations of Zn in plant tissue [note, 'background Zn' refers to Zn in the plant that has not been absorbed via the leaves from foliar fertilisation i.e. Zn taken up via the roots (from the nutrient solution or soil), or Zn originating from the seed)]. This can be problematic as Zn content can vary considerably between plants as previously observed (Read, 2020). Nuclear techniques offer an alternative approach, and have been used to trace the movement of metals in cereals (Erenoglu et al., 2002; Herren and Feller, 1996; Page and Feller, 2015; Pearson and Rengel, 1995). Radiolabelled ^{65}Zn formulations can be used to trace the distribution of foliar-applied ^{65}Zn while avoiding interference from background Zn during analysis; something which is not possible using conventional techniques such as inductively coupled plasma mass spectrometry (ICP-MS). The use of radiolabelled fertiliser allows quantification of absorbed Zn in plant tissue, and in situ visualisation (Read et al., 2019). It remains unknown whether Zn absorbed from foliar-applied ZnO-NPs and ZnO-microparticles (ZnO-MPs) accumulates in wheat grain, or, if previously observed increases in grain Zn are simply a by-product of improved plant nutritional status (i.e. is foliar applied-Zn itself translocated to grain?). Finally, understanding the distribution of Zn in wheat grain is vital for improving the nutritional content of grain. Grain milling removes the outer grain layers, leaving behind the endosperm which is consumed by humans. Therefore, maximising the Zn concentration of the endosperm is essential for improving the nutritional quality of grain. However, the distribution of foliar-applied Zn in wheat grain has not been investigated.

In this study, we carried out a glasshouse experiment with wheat to study the behaviour of foliar-applied ZnO-NPs and ZnO-MPs. To investigate the efficacy of different foliar applications, we used hydroponic growing conditions to ensure that water or other nutrients (apart from Zn) did not have a limiting effect on growth (Poorter et al., 2012), and to tightly control Zn availability. The aim of our study was to compare the efficacy of foliar-applied ZnO-NPs and ZnO-MPs to conventional Zn formulations (soluble Zn and ZnEDTA). We used two different sized particles to test the hypothesis that smaller particles would more easily cross the leaf cuticle. We used radiolabelled ^{65}Zn formulations so we were able to distinguish fertiliser Zn from background Zn in the plant; allowing us to accurately measure the translocation of foliar applied Zn to other plant parts and the grain. $^{65}Zinc$ radiolabelled foliar fertilisers prepared from commercial ZnO-NP formulations (i.e. ^{65}ZnO-NPs were not synthesised using a bottom-up approach) to investigate: (a) Zn mobility and translocation within the plant; (b) grain Zn content; (c) localisation of foliar-applied Zn in grain; (d) optimal application rate; and (e) fertiliser use efficiency. We used gamma spectrometry to quantify the amount of foliar-applied Zn translocated to other plant parts, and photo-stimulated luminescence (PSL) autoradiography to visualise the grain distribution of foliar-applied Zn. This localisation pattern was compared to background Zn (i.e. Zn taken up via the roots from trace amounts of Zn present in the hydroponic solution, or Zn originating from the seed), which was mapped in the same wheat grain using synchrotron-based X-ray fluorescence microscopy (XFM).

2. Materials and methods

A hydroponic glasshouse experiment with wheat was undertaken where four radiolabelled Zn foliar fertilisers were applied to plants, namely ^{65}ZnO-NPs, ^{65}ZnO-MPs, $^{65}ZnEDTA$ and $^{65}ZnCl_2$, at a typical agronomic rate of 750 mg Zn/L (Cakmak and Kutman, 2017). We have previously described the advantages of using radioisotopes over conventional techniques, such as ICP-MS, to evaluate the long-range transport of foliar applied Zn when applied to localised areas on individual leaves (Read et al., 2019). At maturity, plants were harvested, and the plant distribution of foliar-applied ^{65}Zn was determined as well as the localisation of foliar-applied ^{65}Zn in wheat grain. Two lower Zn application rates (75 mg Zn/L and 7.5 Zn mg/L) were also applied to investigate the effect of application rate on grain concentration of foliar-applied Zn.

2.1. Preparation of ^{65}Zn labelled foliar fertilisers

Zinc foliar fertilisers were neutron-activated at ANSTO's OPAL reactor to produce ^{65}Zn labelled fertilisers as described previously (Read et al., 2019). Briefly, approximately 100 mg of each ZnO target (i.e. powder) and 182 mg of the $ZnCl_2$ target were neutron irradiated to achieve a nominal ^{65}Zn activity of each target of 131 MBq (1×10^9 Bq/g Zn). Spiking solutions of ^{65}ZnO-NPs, ^{65}ZnO-MPs, ^{65}ZnEDTA and $^{65}ZnCl_2$ were then prepared from the activated targets at a nominal concentration of 750 mg Zn/L in ultrapure deionised water containing the non-ionic surfactant Triton X-100® (0.05% *v*/v). All Zn treatments were also diluted 10× and 100× with ultrapure deionised water containing Triton X-100 (0.05% v/v) to produce spiking solutions with nominal concentrations of 75 mg Zn/L and 7.5 mg Zn/L. Both ZnO particle suspensions have been characterised pre- and post- neutron irradiation using transmission electron microscopy and dynamic light scattering, where radioabelled ^{65}ZnO-NPs have a diameter of 40–50 nm and ^{65}ZnO-MPs, 300–400 nm (Read et al., 2019). All suspensions were ultrasonicated before leaf application. The dissolution of ZnO-NPs and ZnO-MPs in ultrapure deionised water has been reported previously with 4.4 ± 0.2% and 11.5 ± 0.7% particle dissolution after 24 h for ZnO-MPs and ZnO-NPs, respectively (Read et al., 2020).

2.2. Experimental design and plant growing conditions

Wheat (*Triticum aestivum* cv. Shield) was grown hydroponically in a greenhouse with natural lighting as described previously (Read et al., 2019). Seeds were pre-germinated for 4 d between two sheets of paper towel and kept moist using ultrapure deionised water. Zinc was not added to the hydroponic solution (1/5 strength Hoagland's solution) which comprised 1.0 mM KNO_3, 1.0 mM $Ca(NO_3)_2$, 0.457 mM $MgSO_4$, 0.1 mM KH_2PO_4, 1.0 μM $MnCl_2$, 3 μM H_3BO_3, 1 μM $(NH_4)6Mo_7O_{24}$, 0.2 μM $CuSO_4$, 60 μM Fe(III)-EDTA, 0.0336 mM Na_2SiO_3 and 2 mM 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer (Doolette et al., 2018) and was replaced weekly. The experimental set-up was a randomised complete block design with four replicates for each treatment. The four Zn treatments (ZnO-NPs, ZnO-MPs, $ZnCl_2$ and ZnEDTA) were applied at three concentrations; 750 mg/L (H, high), 75 mg/L (M, medium) and 7.5 mg/L (L, Low), giving a total of 12 treatments, and four control plants that received no foliar Zn, only ultrapure deionised water containing Triton X-100 (0.05% *v*/v).

Ten 5 μL droplets of each foliar fertiliser were applied to the youngest fully emerged leaf (YFEL) 28 d after sowing (28 DAS) at the tillering growth stage (GS21) (Zadoks et al., 1974), providing 37.5 μg of Zn to H plants, 3.75 μg to M plants and 0.375 μg of Zn to L plants nominally. The tiller that contained the treated leaf was designated the 'treated tiller', and all other tillers, were assigned untreated tillers. At maturity (154 DAS), plants were harvested and separated into four plant parts (Fig. S1): 1) stem and leaves from the treated tiller (TT); 2) stem and leaves from the untreated tiller(s) (UTT); 3) grain from the TT (TT-G) and 4) grain from the UTT (UTT-G). Plant parts were dried at 60 °C for 5 d then weighed to determine the effect of Zn treatments on plant biomass and grain yield. The treated leaf was excluded from the analyses due to its high ^{65}Zn concentration; therefore, a complete mass balance was not calculated.

2.3. Quantification of ^{65}Zn in plant parts at maturity

Once absorbed by the plant, the translocation of foliar-applied Zn was determined by measuring the ^{65}Zn activity of all plant parts. Samples were homogenised to a find powder then the ^{65}Zn activity measured using gamma spectrometry. For stems and leaves, this was performed using a ball mill (Retsch), whereas for grain we used a domestic grinder (Sunbeam Multigrinder – EM0405) followed by a homogenizer (Polytron® Kinematica AG Polytron PT 3000). Mills and grinders were cleaned with ethanol (96%) and deionised water between each sample to eliminate cross-contamination. ^{65}Zinc activity was determined from approximately 1 g of homogenised sample in glass tubes (Chase Scientific). Samples were analysed using an automated gamma counter (Wallac Wizard 1470) as described previously using in-house prepared liquid standards of known ^{65}Zn activity in the same volume as samples (Read et al., 2019). The proportion of applied Zn in a particular plant part was calculated as follows:

$$\textit{Proportion of total applied Zn in plant part } (\%) = \frac{M_{PP}}{M_{fertiliser}} \quad (1)$$

where, $M_{fertiliser}$ is the total mass of Zn applied to the plant and M_{PP} is the mass of applied Zn (ng) in a plant part and where,

$$M_{PP} = C_{PP} \times DW_{PP} \quad (2)$$

and C_{PP} is the concentration of applied Zn in a plant part (ng applied Zn/g) calculated using Eq. (3), and DW is the yield of that plant part (dry weight, g).

$$C_{PP} = \frac{\left(\frac{A_{sample}}{SA_{fertiliser}}\right) \times 1x10^9}{DW_{sample}} \quad (3)$$

where A_{sample} is the activity of the sample (Bq), and $SA_{fertiliser}$ is the specific activity of the Zn fertiliser (Bq/g), and DW_{sample} the mass of analysed plant sample (g). All radioanalyses were corrected for background radiation and physical decay.

2.4. Distribution of foliar-applied Zn in wheat grain

The distribution of foliar-applied Zn in wheat grain was visualised using PSL autoradiography, and compared to the distribution of Zn taken up from the roots or supplied from the seed using synchrotron-based X-ray fluorescence microscopy (XFM). For both distribution studies the same transverse and longitudinal thin-sections of wheat grain were analysed. Grain collected from the treated tiller of plants treated with ZnO-NPs, ZnO-MPs, ZnEDTA and $ZnCl_2$ at the highest application rate (750 mg/L), were mounted on a Menzel 75 mm by 25 mm (glass) microscope slide using superglue (Loctite 401). Grain was then embedded in resin (Araldite GY 191 Huntsman) and left to cure for 24 h. Thin-sections (≈210 μm) of resin embedded grain were then obtained at the desired thickness by hand making the section and polished with lapping film (Starcke 991A silicon carbide paper 1200 girt and other grit sizes). To avoid sample contamination, each sample was polished on a different piece of lapping film.

2.4.1. Localisation of ^{65}Zn in wheat grain using autoradiography

The distribution of ^{65}Zn in wheat grain was visualised using autoradiography. The lapped grain sections were placed in a standard radiographic imaging cassette, covered by a 3.6 μm Mylar film and exposed to a BAS-SR 2050 phosphor plate (Fuji Film). The exposure was stopped after 2 h and the resulting photo-stimulated luminescence visualised using a FLA 7000 scanner (GE). The resulting image was processed using Fiji (Schindelin et al., 2012).

2.4.2. Elemental Zn mapping using synchrotron-based X-ray fluorescence microscopy

The localisation of total Zn in wheat grain (i.e. background plus foliar application) – from roots, seed and foliar translocation – was analysed using synchrotron-based XFM. The same wheat thin-sections used for autoradiography were floated off the microscope slide, and the resin dissolved, by submerging the slide in 99.5% acetone for 12 h. The thin-sections were mounted on a 10 cm × 10 cm sample holder between two pieces of ultrathin (4 μm) Ultralene® film. The XFM mapping was carried out at the XFM beamline of the Australian Synchrotron (ANSTO) in Melbourne, Victoria. The incident beam was set at

18.5 keV using a Si(111) monochromator, and focused to approximately 2 μm × 2 μm using Kirkpatrick-Baez mirrors (Paterson et al., 2011). The XRF signal emitted by the sample was collected using a 384-element Maia detector in backscatter geometry. The sample was scanned on-the-fly in the horizontal direction with discrete steps in the vertical direction. The sampling interval was 0.002 mm with a vertical step of 0.002 mm, and dwell time for each pixel was 0.4 ms. The XFM data were analysed using GeoPIXE (Ryan, 2000; Ryan and Jamieson, 1993)

2.5. Statistical analyses

Both the normality of distribution and constant error variance assumptions were tested for each analysis. Statistical analyses were performed using analysis of variance (ANOVA) and paired *t*-tests in GraphPad Prism (version 8.2.0). Differences between treatments were determined at the 5% significance level using Fisher's Protected L.S.D.

3. Results and discussion

3.1. Effect of Zn foliar fertilisers on plant biomass and grain yield

The total plant biomass and grain yield of all Zn treatments applied at 750 mg Zn/L was measured at maturity (154 DAS; Fig. 1). Each plant was divided into four parts; stem and leaves of the Zn treated tiller (TT) and untreated tiller(s) (UTT), and grain from the treated and untreated tillers (TT-G and UTT-G, respectively). For all Zn treatments, the biomass of UTT was greater than TT as UTT consisted of two or more tillers whereas TT was a single tiller only. In terms of total plant biomass (TT + UTT + TT-G + UT-G) there were no significant differences between Zn treatments ($p = 0.1424$), or from the control plants i.e. plants that received no foliar Zn (Fig. 1). Although this difference was not significant, the total plant biomass of plants treated with Zn at the highest application rate (750 μg/L), did increase in the order $ZnCl_2$ < ZnEDTA < ZnO-MP < ZnO-NP. We did not observe any visual signs of severe Zn deficiency in any plants, including the control plants. However, between the mid-vein and edge of the middles leaves of some plants we did observe pale patches. This effect appeared randomly (i.e. regardless of Zn treatment type of application rate), and could indicate Zn deficiency.

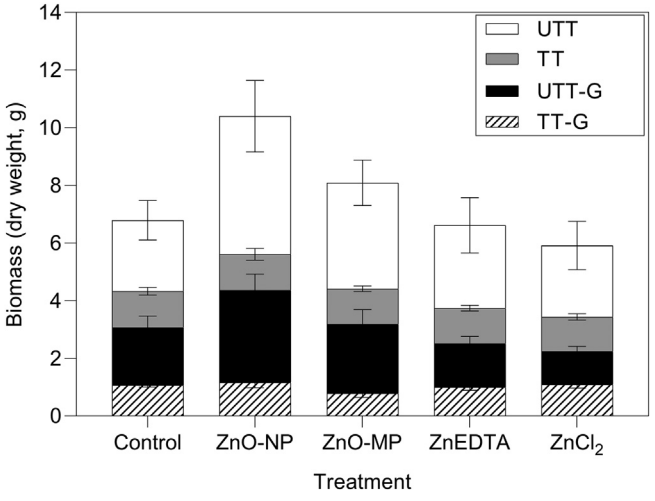

Fig. 1. Mean biomass (dry weight, g) of wheat plants harvested at maturity. Zinc was applied at 750 mg Zn/L. Plants were separated into stem and leaves from the Zn treated tiller (TT) and untreated tiller(s) (UTT), and, grain from these tillers (TT-G) and (UTT-G). The means for each Zn treatment (Zn-oxide nanoparticles [ZnO-NP], microparticles [ZnO-MP], chelated Zn [ZnEDTA], soluble Zn-chloride [ZnCl2] and control [surfactant and ultrapure deionised water]) are averaged from four plants. Error bars correspond to standard error. There were no significant differences in total plant biomass between Zn treatments, or from the control, based on one-way ANOVA ($p = 0.1424$).

Excluding grain, there was also no significant difference in the biomass of stems and leaves (TT + UTT) between Zn treatments, or from the control ($p = 0.404$). Given that Zn was only applied to one leaf, we did not expect an effect on vegetative growth. As our study was primarily a ^{65}Zn radiotracing study to investigate the distribution and translocation of Zn, we only applied Zn fertiliser to one leaf on each plant. This is in contrast to field applications where foliage coverage with Zn is near complete. Therefore, although the Zn concentration of our fertilisers was typical of agronomic solutions – or even less than some recommended doses (1500 mg/L) – the total mass of Zn applied to each plant was substantially less than what would occur in the field. Therefore, any stimulatory effect on plant biomass as a result of increased Zn plant content was likely to be minimal.

Significantly more grain (TT-G + UTT-G) was produced in ZnO-NP treated plants (mean = 4.36 g, $n = 4$) compared to $ZnCl_2$ plants (2.24 g, $n = 4$) ($p = 0.0216$), but there were no significant differences between any other Zn treatments ($p > 0.05$). It is unknown why grain yield increased in ZnO-NP plants. These plants did have more vegetative growth than other Zn treatments, although not significantly more, which may explain the higher grain yield for this treatment.

Typically, the nutrient status of the plant dictates if a Zn response will be observed following Zn foliar fertilisation. This response is usually seen as an increase in grain Zn concentration rather than an increase in grain yield (Norton, 2014; Peck et al., 2008). Increases in grain yield following the foliar application of Zn typically only occurs when the plant is Zn deficient (Kopittke et al., 2019). Plants in the current study were under Zn stress as Zn was only supplied to plants as foliar fertilisers and no additional Zn was added to the growing medium. While this may explain the grain yield response for ZnO-NP plants, it does not explain why grain yield did not increase for any of other Zn treatments compared to the control plants.

Although a number of recent studies have compared the effects of soil applied ZnO-NPs and soluble Zn formulations on wheat grain yield, few have investigated the foliar application of these formulations and subsequent effects on grain yield. In field experiments, Zhang et al. (2018) found that foliar application of uncoated ZnO-NPs (hydrodynamic diameter, $d_h = 406$ nm) at 2000 mg/L, and $ZnSO_4$ at 7000 mg/L, to winter wheat did not increase grain yield. To our knowledge, this is the only study to have reported the effects of foliar ZnO-NP on wheat grain yield. Other studies have investigated foliar application of ZnO-NPs to other plant species. In field-grown foxtail millet, foliarly applied ZnO-NPs (17.3 ± 0.1 nm) did not increase grain yield compared to untreated plants (Kolenčík et al., 2019). This study differed from our experiments in that a different plant species was used, and Zn was applied twice during the growing season. In addition, ZnO-NP fertiliser was applied at a low concentration of 2.6 mg/L. Therefore, the lack of effect on grain yield is not unexpected.

3.2. Distribution of foliar-applied Zn in mature wheat plants and translocation to grain

The translocation of absorbed foliar-applied ^{65}Zn (applied at 750 mg Zn/L) was determined using gamma spectrometry (Fig. 2). Mature plants were again separated into four parts: grain collected from the untreated and Zn treated tillers (UTT-G and TT-G, respectively), and stem and leaves from these tillers (UT and TT, respectively). As the focus of our study was to investigate the translocation of foliar-applied Zn, the leaf treated with ^{65}Zn was excluded from our data analyses. We have previously found that for very soluble Zn foliar fertilisers, such as $ZnCl_2$, nearly 100% of applied Zn is internalised within the leaf tissue after 14 d (Read, 2020). Therefore, the treated leaf was removed from the TT and UTT analyses. By internalised, we are referring to Zn that has been absorbed by the leaf and is present in the plant tissue i.e. not Zn that is adsorbed on the leaf surface. In a previous study, we were

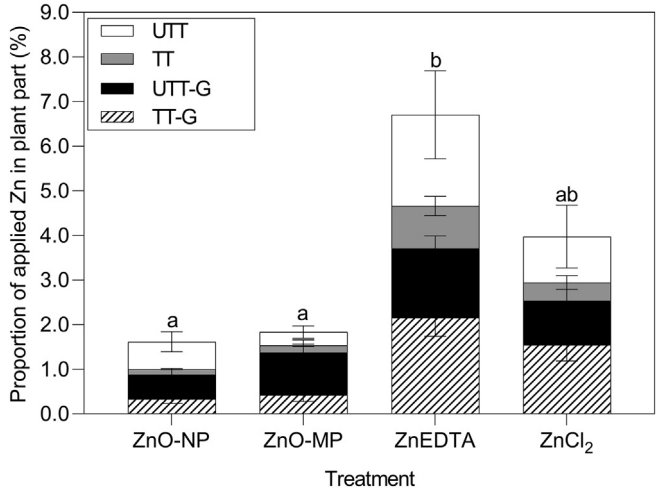

Fig. 2. Distribution of foliar-applied Zn in wheat plants at maturity. Zinc was applied at 750 mg Zn/L. The mean ($n = 4$) proportion of applied Zn in the stems and leaves of the untreated tiller (UTT) and treated tiller (TT), and corresponding grain (UTT-G and TT-G, respectively) are shown. Letters indicate significant differences between Zn treatments for the total proportion of applied Zn in plants ($p = 0.0166$) according to one-way ANOVA with Fisher's Protected LSD test.

able to distinguish between internalised and externalised Zn using XFM analysis where we first validated our leaf washing technique (Doolette et al., 2018) to confirm that surface adsorbed Zn had been removed.

Zinc-EDTA treated plants had the highest proportion of applied Zn in aboveground plant tissue (6.3%, $n = 4$) (all above ground biomass was measured excluding the treated leaf and chaff). This was significantly higher than the proportion of applied Zn measured in ZnO-NP (1.6%, $n = 4$, $p = 0.0240$) and ZnO-MP (1.7%, $n = 4$, $p = 0.0287$) plants, but was not significantly different to $ZnCl_2$ plants (3.9%, $n = 4$, $p = 0.369$). For plants treated with $ZnCl_2$, the most soluble Zn formulation, although the total proportion of applied Zn in plant tissue was higher than ZnO-NP and ZnO-MP plants, the differences were not significant ($p = 0.365$ and $p = 0.416$, respectively). There was also no significant difference ($p > 0.05$) between ZnO treatments. This suggests that ZnO-NP/MP formulations are as efficacious, in terms of Zn translocation, as commonly used soluble Zn formulations. And, that Zn supplied as ZnEDTA has much greater mobility in plant tissue.

The results support our previous work which showed that more Zn is translocated to new plant tissue when foliar Zn is applied as ZnEDTA than as ZnO-NPs or ZnO-MPs (Read et al., 2019). Specifically, in a short term uptake study where Zn was applied to one leaf at the same spiking concentration as the current study (750 mg/L), 0.5% of Zn applied as ZnEDTA was detected in the new leaf after 14 d, whereas for ZnO-NPs only 0.1% of applied Zn was translocated, and 0.06% for ZnO-MPs.

Focusing on grain, the highest proportion of Zn translocated to grain occurred in ZnEDTA plants, where 3.3 ± 0.5% of applied Zn was present in the grain (Fig. 2). This was significantly higher ($p = 0.008$) than ZnO-NP and ZnO-MP treatments, where only 0.8 ± 0.1% and 1.3 ± 0.3% of applied Zn, respectively, was measured in grain. Zinc chloride treated plants were intermediate, with 2.5 ± 0.5% of applied Zn translocated to grain, which was not significantly different from either ZnEDTA, or the ZnO treatments.

Our results are in contrast with the only other comparable study using wheat (Zhang et al., 2018), where foliar application of ZnO-NPs increased Zn grain concentration more than $ZnSO_4$ application (Zhang et al., 2018). This could be due to differences between the two studies including a) plant physiology between different cultivars which may affect Zn demand and cuticle properties (e.g. cuticle thickness, and stomata and trichome density) b) NP size which affects the adhesion and rate of Zn uptake by the leaf (Avellan et al., 2019) c) chemical properties of $ZnCl_2$ and $ZnSO_4$ [e.g. point of deliquescence ($ZnCl_2$: 10% (Shoji and Ohnaka, 1989) and $ZnSO_4$: 90% (Pilinis et al., 1989)) and solubility ($ZnCl_2$: 432 g/100 mL and $ZnSO_4$: 57 g/100 mL in water at 25 °C (Merck and Co Inc, 2001))] and d) growing conditions (field vs hydroponic greenhouse, where more Zn would be taken up by roots in the field compared to the current study where Zn was not added to the growing medium). While previous ZnO-NP foliar fertiliser studies provide extremely useful information on the efficiency of such fertilisers for increasing total grain Zn concentration, ours is the first study to use ^{65}Zn radiolabelled ZnO-NPs to trace the translocation and distribution of foliar-applied ZnO-NP in plants. Therefore, rather than presenting data for total grain Zn concentrations – which can only be determined by subtracting background Zn levels from separate control plants – we were able to accurately determine the amount of foliar-applied Zn translocated to grain without interference from non-foliar applied Zn.

The mobility of ZnO-NP foliar fertilisers to grain/fruit in other crop species has been investigated. In pinto bean (*Phaseolus vulgaris*), foliar application of 30 nm ZnO-NPs at 1000 mg Zn/L increased Zn seed content significantly more than $ZnSO_4$ and chelated Zn formulations applied at 0.3% *w/v* ($p < 0.01$) (Mahdieh et al., 2018). Similarly, in coffee plants (*Coffea Arabica* L.), ZnO-NP treated leaves ($d_h = 621$ nm) had a higher Zn content than plants treated with $ZnSO_4$ (1267 vs 344 mg/kg DW) at the same Zn concentration (10 mg/L) (Rossi et al., 2019). However, the rinsing technique used to remove unabsorbed Zn from the leaf surface was not validated in that study (Rossi et al., 2019). Therefore, the higher concentration of Zn in ZnO-NP treated leaves could be due to incomplete removal of unabsorbed Zn from the leaf surface, leading to an overestimation of in situ Zn. Given that ZnO is considerably less soluble than $ZnSO_4$, residual Zn is more likely on the surface of ZnO-NP treated leaves when leaves are washed with water only as occurred in that study.

When comparing the grain data for untreated and treated tillers (Figs. 2 and 7), a greater proportion of applied Zn was present in the treated tiller of ZnEDTA and $ZnCl_2$ plants, whereas for ZnO-NPs and ZnO-MPs, the untreated tillers contained more applied Zn. This may suggest that when Zn is applied in particulate form as either ZnO-NP or ZnO-MPs, it is more mobile in plant tissue and translocates from the tiller to which it was applied. However, the more likely explanation is a dilution effect. Although not significant, grain yield was higher in the untreated tillers of ZnO-NP and ZnO-MP plants than the treated tillers, whereas for $ZnCl_2$ and ZnEDTA, grain yield was comparable from both tillers (Fig. 1). A similar effect was observed by McDonald et al. (2008) who investigated the relationship between grain Zn concentration and grain yield in field and pot studies with bread wheat. The authors demonstrated an inverse relationship between grain yield and grain Zn concentration, which they attributed to a dilution effect, specifically, the higher number of kernels produced when grains had a higher Zn concentration.

3.3. Grain distribution of Zn and foliar-applied Zn using X-ray fluorescence microscopy (XFM) and PSL autoradiography

To our knowledge, this is the first time that the distribution of foliar-applied Zn has been examined in wheat grain in situ. Autoradiography of transverse (Fig. 3e–h) and longitudinal (Fig. 4e–h) wheat thin-sections was used to determine the distribution of foliar-applied ^{65}Zn in grain, following its application at 750 mg Zn/L. Its distribution was then compared to that of total grain Zn (background Zn + applied Zn), in the same sections, which was mapped using synchrotron-based XRF (Figs. 3a–d and 4a–d). For ZnEDTA and $ZnCl_2$ treatments, the higher intensity of the ^{65}Zn signal was observed in the crease region and aleurone layer, with the crease region having a greater signal intensity (Fig. 3e, f). Foliar ^{65}Zn was also homogenously distributed throughout the endosperm for both treatments (Figs. 3e, f and 4e, f). In transverse sections, it can be seen that ^{65}Zn accumulated in the embryo for ZnEDTA and $ZnCl_2$ treated plants, and was also present in the

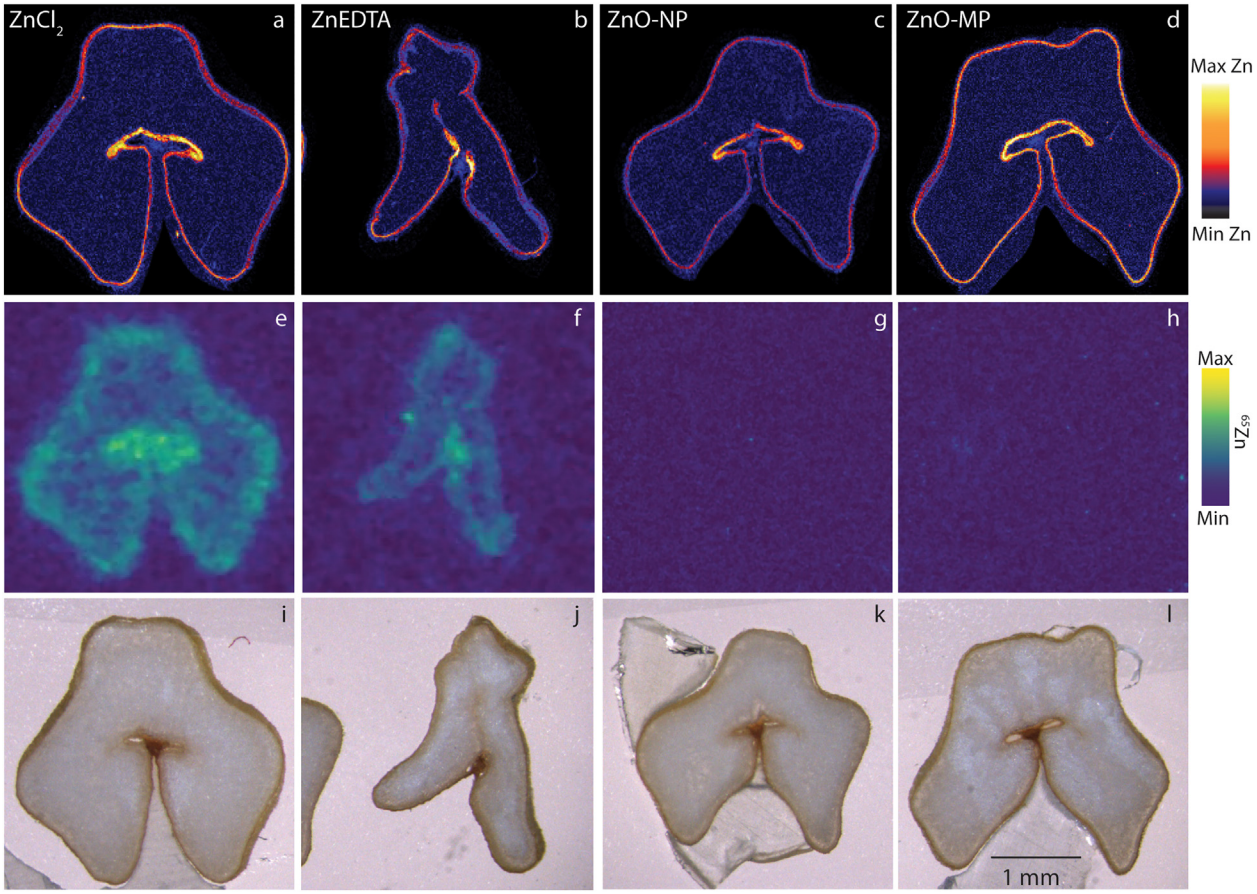

Fig. 3. Distribution of total Zn (*a-d*; XFM) and ^{65}Zn (e–h; autoradiography) in transverse thin-sections of wheat grain. Grains were collected from the tiller containing the leaf treated with foliar Zn as either Zn chloride ($ZnCl_2$; a, e, i), ZnEDTA (b, f, j), Zn oxide nanoparticles (ZnO-NP; c, g, k) or ZnO microparticles (ZnO-MPs; d, h, l). The corresponding light microscope image of each grain thin-section (210 μm thickness) is shown in *i-l*.

aleurone layer and endosperm (Fig. 4e, f). For both ZnO particle treatments, there was insufficient ^{65}Zn to clearly visualise the distribution of foliar-applied Zn in transverse sections (Fig. 3g, h). In longitudinal sections, although the amount of ^{65}Zn is too low to clearly see its distribution in the grain, its presence in the embryo is evident (Fig. 4g, h).

X-ray fluorescence maps (Figs. 3a–d and 4a–d) show the distribution of all stable and radioactive Zn isotopes (i.e. total Zn). Therefore, the Zn grain maps (Figs. 3a–d and 4a–d) show the distribution of stable Zn absorbed via the roots and from the seed (i.e. background), as well as stable Zn and ^{65}Zn translocated from leaves following foliar application

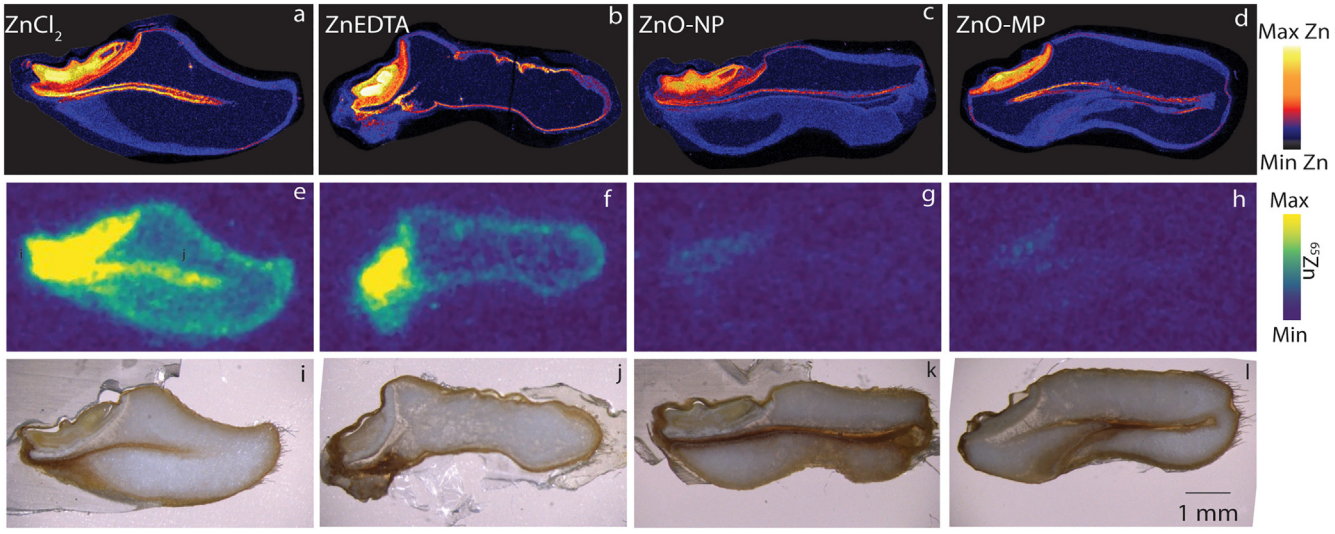

Fig. 4. Distribution of total Zn (*a-d*) and ^{65}Zn (e–h) in longitudinal thin-sections of wheat grain. Grains were collected from the tiller containing the leaf treated with foliar Zn as either Zn chloride ($ZnCl_2$; a, e, i), ZnEDTA (b, f, j), Zn oxide nanoparticles (ZnO-NP; c, g, k) or ZnO microparticles (ZnO-MPs; d, h, l). The corresponding light microscope image of each grain thin-section (210 μm thickness) is shown in i-l.

of the radiotracer. The XRF signal is dominated by stable Zn isotopes, not ^{65}Zn: at the time of fertiliser application, 3×10^{-4}% of Zn in the Zn fertilisers was labelled with ^{65}Zn, which decreased to 5×10^{-5}% at the time of XFM analysis following the radioactive decay of ^{65}Zn to ^{65}Cu. The grain distribution of total Zn was comparable to that of foliar-applied Zn (Figs. 3e, f and 4e, f) and was similar for all Zn treatments. Zinc was highest within the crease region, aleurone layer and embryo. This is in agreement with previous studies which have shown that Zn accumulates in these regions following foliar application of $ZnSO_4$ (Ajiboye et al., 2015; Zhang et al., 2018) and ZnO-NPs (Zhang et al., 2018), and that the Zn concentration of the aleurone layer (100 mg/kg) can be up to ten times that of the endosperm (10 mg/kg) (Ozturk et al., 2006). While these studies have provided valuable information regarding Zn grain distribution they were not able to distinguish between background Zn and foliar-applied Zn.

Understanding the distribution of Zn in wheat grain is vital for improving the nutritional content of grain. Grain milling and grain processing removes the outer grain layers including the aleurone layer and embryo, leaving behind the endosperm which is consumed by humans. Accordingly, maximising the Zn concentration of the endosperm is essential for improving the nutritional quality of grain. In wheat field trials, two foliar applications of $ZnSO_4$ during booting and milk stages significantly increased endosperm Zn concentrations from 8 to 15 mg Zn/kg ($p < 0.05$) (Cakmak et al., 2010). However, that study used mechanical fractionation to analyse grain parts, not in situ analysis. Based on our autoradiography images, the distribution of foliar-applied Zn appears to mirror the distribution of non-foliar applied Zn regardless of the form in which it is applied. This supports our hypothesis that grain Zn distribution would not be affected by the form in which it is applied to leaves. Importantly, there appears to be no difference in localisation between Zn treatments. Autoradiography imaging and XFM maps suggest that foliar ZnO particulate treatments do not afford any benefit, or disadvantage, in terms of Zn localisation in wheat grain. And, the grain distribution of Zn is the same regardless of whether Zn is taken up via the roots (or from the seed) or from Zn-fertilised foliage.

3.4. Grain yield is not affected by foliar Zn application rate

To investigate the effect of Zn application rate, all foliar Zn fertilisers were applied at two lower concentrations; 75 mg/L (medium, M) and 7.5 mg/L (low, L). Total average grain yield of M and L plants was compared to plants that received the highest Zn application rate (H, 750 mg/L) (i.e. Sections 3.1–3.4).

Within each Zn treatment, total grain yield was not significantly different between application rates, or from the control ($p > 0.05$; Fig. 5). In fact, when ZnEDTA and $ZnCl_2$ were applied at the lowest application rate (L), average grain yield (4.7 g and 4.4 g, respectively, $n = 4$ plants) was higher than in the M plants (2.6 g and 3.2 g, respectively, n = 4 plants), and H plants (2.5 g and 2.4 g, respectively, $n = 4$ plants). These differences however were not significant ($p = 0.113$ for ZnEDTA and $p = 0.146$ for $ZnCl_2$). The same trend was observed in ZnO-MP plants, where average grain yield was 4.5 g at the medium application rate, and 3.2 g at the highest application rate ($n = 4$ plants). Again, these differences were not significant ($p = 0.364$). Zinc-oxide NP fertiliser was the only treatment where the highest application rate produced the highest grain yield (4.6 g vs 3.4 g for M plants), although not significantly different ($p = 0.244$).

Our results are in agreement with most previous studies, in particular large-scale field trials, which have shown that foliar fertilisation of wheat with conventional Zn formulations (i.e. $ZnSO_4$) does not increase grain yield (Ram et al., 2016; Zhang et al., 2018; Zou et al., 2012). Similar results have been shown in other major crop species, such as field-grown rice (*Oryza sativa* L.), where foliar application of Zn as $ZnSO_4$ at 5000 mg/L did not affect yield regardless of the time of application (Yang et al., 2011).

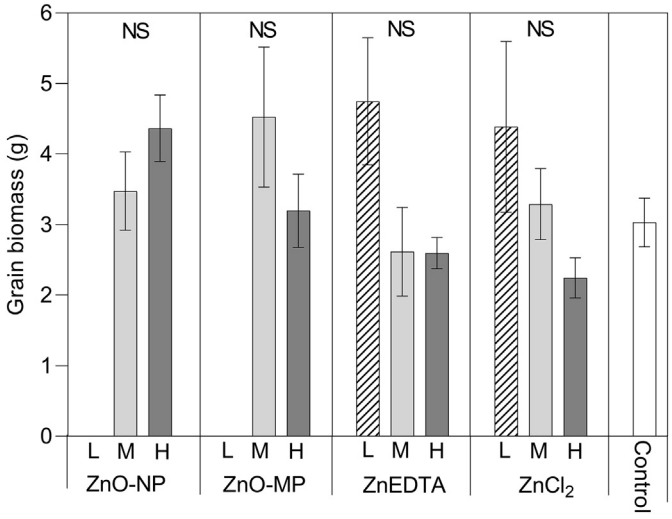

Fig. 5. Mean grain biomass (dry weight, g) of plants treated with ZnO-NP, ZnO-MP, ZnEDTA or $ZnCl_2$ at low (L, 7.5 mg Zn/L), medium (M, 75 mg Zn/L) and high (H, 750 mg Zn/L) Zn nominal concentrations. The mean for each treatment is the average of four plants. The error bars indicate standard error. No Zn treatments were significantly different from the control (NS) at 0.05 significance level based on one-way ANOVA (ZnO-NP, $p = 0.2155$; ZnO-MP, $p = 0.3311$; ZnEDTA $p = 0.1126$; $ZnCl_2$, $p = 0.1459$). 'L' treatments for ZnO-NP and ZnO-MP were not analysed as the ^{65}Zn concentration was below the detection limit.

3.5. Effect of Zn application rate on the grain concentration of applied Zn

The plant distribution and grain concentration of absorbed Zn (ng applied Zn/g) at lower application rates was quantified using gamma spectrometry (Fig. 6). The average grain concentration was calculated by averaging the UTTs and TTs of each plant ($n = 4$ plants). For both ZnO fertilisers, grain concentration was not affected by Zn application rate ($p > 0.05$). Note, at the lowest application rate (L-ZnO-NP and L-ZnO-MP), grain concentration of applied Zn was below the limit of detection i.e. 10 ng applied Zn/g, which equates to an activity of 5 Bq at the time of analysis with a sample mass of 1 g. For ZnEDTA and $ZnCl_2$

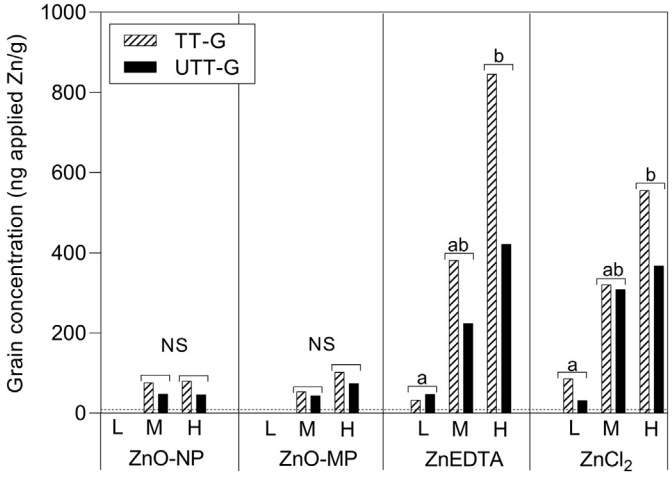

Fig. 6. Grain concentration of applied Zn. Zinc treatments (ZnO-NP, ZnO-MP, ZnEDTA or $ZnCl_2$) were applied to one leaf (i.e. the treated tiller, T) at low (L, 7.5 mg Zn/L), medium (M, 75 mg Zn/L) and high (H, 750 mg Zn/L) Zn concentrations. Grain collected from wheat heads on the tiller that was not treated with Zn (U) is indicated by filled bars. Grain from L-ZnO-NP and L-ZnO-MP were below the limit of detection (10 ng applied Zn/g) which is shown by the dashed line. Letters indicate significant differences between mean grain concentrations ($n = 8$) for each application rate based on one-way ANOVA and Fisher's Protected LSD test at 95% significance. NS: no significant difference.

plants, the average grain concentration of H plants was significantly higher than plants that received 100× less Zn (L plants) ($p = 0.0197$ for ZnEDTA and $p = 0.0456$ for $ZnCl_2$). However, for both Zn treatments, plants that received the intermediate Zn concentration (M) were not significantly different from the low or high application rates. Despite there being a decreasing trend in grain concentration with decreasing application rate, the relationship was not proportional.

The grain concentration of applied Zn does not provide information on fertiliser efficiency. To compare the efficiency of each treatment, the total amount of Zn applied to each plant was considered together with grain biomass to give the total proportion of applied Zn translocated to the grain. Quite a different picture emerges when this analysis is performed (Fig. 7). Focusing on the conventional fertilisers first, when ZnEDTA and $ZnCl_2$ were applied to leaves at the lowest application rates, significantly more Zn was transported to grain. For example, in H-$ZnCl_2$ plants, only 2.5% of applied Zn was found in the grain, whereas at the lower applications rates, significantly more ($p = 0.0001$) applied Zn was measured in the grain (30.2% for M and 25.6% for L). A similar trend was observed for ZnEDTA, with only 3.3% of Zn present in grain at the highest application rate, and significantly more for M (20.3%) and L (26.8%) ($p = 0.0009$) plants. For both ZnO treatments, Zn application at the medium rate also resulted in significantly more applied Zn in the grain than the highest rate (6.0% vs 0.8% for ZnO-NP, and 10.1% vs 1.4% for ZnO-MP).

It is unclear why lower application rates increased the proportion of applied Zn translocated to grain. One possibility is that at high concentrations, Zn foliar sprays had a toxic effect, evidence by leaf scorch (Fig. S2), which inhibited Zn^{2+} transport within the plant. We have previously shown that Zn^{2+} absorbed by leaves treated with $ZnSO_4$ or ZnEDTA at 750 mg/L is complexed by organic and inorganic ligands (Zn-phytate and Zn-phosphate, respectively) in leaf tissue, and propose that this may act as a detoxification mechanism to decrease the mobility and bioavailability of Zn^{2+} (Doolette et al., 2018). Similarly for soil applications of $ZnSO_4$ and ZnO-NPs, it has been observed that $ZnSO_4$ is more toxic with greater inhibitory effects on root and shoot length, and seed germination (Du et al., 2019).

To our knowledge, no data exist on the effect of different Zn application spray concentrations on Zn grain content. However, in corn (*Zea mays* L.) field trials on a Zn deficient soil, Drissi et al. (2015) applied

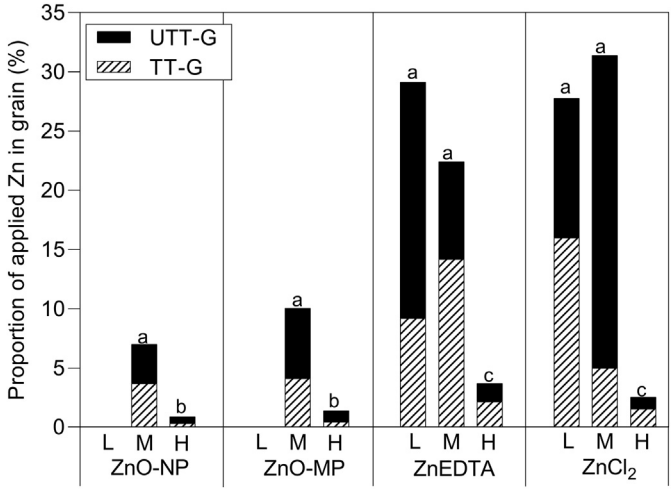

Fig. 7. Translocation of foliar-applied Zn to wheat grain for plants treated with ZnO-NP, ZnO-MP, ZnEDTA or $ZnCl_2$ at low (L, 7.5 mg Zn/L), medium (M, 75 mg Zn/L) and high (H, 750 mg Zn/L) Zn concentrations. Mean ($n = 4$) proportion of applied Zn in grain collected from the Zn treated tiller (T) and untreated tiller (U). Letters indicate significant differences rates for a single Zn treatment based on paired *t*-tests for ZnO-NP ($p = 0.0373$) and ZnO-MP ($p = 0.0067$) and one-way ANOVA and Fisher's Protected LSD test for ZnEDTA ($p = 0.0009$) and $ZnCl_2$ ($p = 0.0001$). Grain from L-ZnO-NP and L-ZnO-MP were below the limit of detection.

Zn foliar sprays at five concentrations (0.03% to 18% *w*/*v*) and found that 0.09% was the optimum concentration for overcoming Zn deficiency while avoiding the negative effects observed at the higher application rates (plant growth inhibition and decrease in plant nutrient composition). In corn field trials in Zn sufficient soil where ZnEDTA and $ZnSO_4$ were applied foliarly at three concentrations (0.56, 1.12 and 2.24 kg Zn/ha), the Zn tissue content of ZnEDTA treated plants was significantly higher at the highest application rate, whereas for $ZnSO_4$, there was no difference between the medium and high application rates (Golden et al., 2016).

3.6. A greater proportion of foliar applied Zn is translocated to grain at lower Zn application rates

Our results suggest that for wheat grown under Zn-deficient conditions, foliar Zn sprays applied at 750 mg/L are less efficient than lower application rates. The relationship between Zn application rate and grain Zn concentration was not proportional for any Zn treatment. For example, when plants received 37.5 μg of Zn as ZnEDTA, the resulting average grain concentration (633 ng applied Zn/g, $n = 8$) was only double the grain concentration (302 ng applied Zn/g) of plants that received 10× less Zn (3.75 μg). This was also the case for $ZnCl_2$ applications. For both ZnO particulate fertilisers, there were no significant differences in Zn grain concentration between medium and high application rates. Therefore, our results suggest that regardless of the form in which foliar Zn is applied to wheat, lower application rates could be used to increase Zn fertiliser use efficiency. Furthermore, the concentration of foliar Zn sprays that are currently used (1000 mg/L), are likely to be higher than required for optimal fertiliser efficiency. This has important implications for growers as it may represent a significant cost saving. We recommend carrying out field trials using the same application rates to confirm these findings: our study has several limitations including that plants were grown in hydroponic conditions, and, Zn was applied to one leaf rather than complete foliar coverage.

Production costs for grain producers are not uniform, either globally or regionally. For example, in Australia, variable costs (input costs) for wheat range from approximately 220 AUD/ha in low rainfall regions up to 620 AUD/ha in high rainfall zones (Rural Solutions, 2019). Despite this variation in expenditure, fertiliser is the largest single variable cost (up to 25%) (Christy et al., 2015; IPNI, 2013). While most countries record the consumption of macronutrient fertilisers (N,P,K), data collection for micronutrients (e.g. Zn) is less common. In fact, from the mid-1980s, the United States and Australia both ceased recording Zn fertiliser consumption (Mortvedt and Gilkes, 1993). Therefore, we recommend performing a cost-benefit analysis of applying foliar Zn at 10-fold and 100-fold lower concentrations than current practice.

4. Conclusion

In this study, the efficacy of foliar-applied ZnO-NPs and ZnO-MPs in wheat was investigated using ^{65}Zn radiolabelled fertilisers, and compared to that of $ZnCl_2$ and ZnEDTA. We investigated the effect of foliar Zn fertilisers on Zn translocation, grain yield and grain Zn content at three Zn application rates; 7.5 mg/L, 75 mg/L and 750 mg/L. At the highest application rate, Zn treatments did not affect grain yield. However, when Zn was applied as ZnEDTA, more Zn was translocated to plant tissue (6.7%) compared to $ZnCl_2$ (3.9%), ZnO-NPs (1.6%) and ZnO-MPs (1.8%) treatments. In terms of grain Zn, the greatest proportion of applied Zn was found in ZnEDTA (3.3%) and $ZnCl_2$ (2.5%) grain, with significantly less Zn in ZnO-NP grain, where only 0.8% of applied Zn was detected. Autoradiography imaging and synchrotron-based XFM mapping showed that the localisation of foliar-applied Zn in grain mirrors that of Zn taken up from the roots or originating from the seed, with accumulation in the embryo, aleurone layer and crease region, and less Zn in the endosperm. This demonstrates that irrespective of whether Zn is taken up from the roots or foliage, the grain

distribution of Zn is the same. Therefore, soil and foliar Zn fertilisers will have the same benefit to the nutritional content of grain, in terms of grain distribution of Zn, which is important for human nutrition following grain processing.

For both ZnO particle treatments, application rate did not affect either grain yield or the translocation of fertiliser Zn to grain. In fact, for all Zn treatments, when 10-fold less Zn was applied (37.5 μg vs. 3.75 μg), the grain concentration of applied Zn was not significantly different. It follows that lower Zn application rates appeared more efficient, particularly for $ZnCl_2$ and ZnEDTA: more than 20% of applied Zn was found in the grain of plants that received Zn at 75 mg/L and 7.5 mg/L, whereas for 750 mg/L applications, <5% of applied Zn was detected in grain. There is potential that the concentration of Zn in foliar fertilisers can be significantly reduced for application to Zn-deficient wheat while not compromising the Zn nutritional quality of grain; potentially offering significant economic advantages to growers and farmers. Future research should focus on larger scale field trials that provide total foliar Zn coverage. Results from such studies can be used to optimise the application rates of chelated and inorganic Zn foliar fertilisers, and, to investigate the efficiency of ZnO particulate fertilisers under field conditions.

CRediT authorship contribution statement

C. Doolette: Conceptualization, Methodology, Formal analysis, Investigation, Writing - original draft, Visualization, Funding acquisition, Project administration. **T. Read:** Conceptualization, Methodology, Investigation, Writing - review & editing. **N. Howell:** Methodology, Investigation, Writing - review & editing. **T. Cresswell:** Conceptualization, Resources, Investigation, Methodology, Writing - review & editing, Formal analysis. **E. Lombi:** Conceptualization, Supervision, Project administration, Funding acquisition, Methodology, Investigation, Writing - review & editing.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2020.142369.

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