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REGULAR ARTICLE

X-ray fluorescence microscopy of zinc localization in wheat grains biofortified through foliar zinc applications at different growth stages under field conditions

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Abstract
Aim Biofortification of wheat with zinc (Zn) through foliar Zn application has been proposed as an agronomic strategy to increase grain Zn concentration, which could serve as a nutritional intervention in regions with dietary Zn deficiency.
Methods Bread wheat (*Triticum aestivum L.*) was biofortified through foliar Zn applications at different growth stages. The concentration of Zn and associated micronutrient in harvested whole grains was determined by ICP-OES. Synchrotron-based X-ray fluorescence microscopy (XFM) was then used to investigate the localization of Zn and associated micronutrients in cross sections of these grains.
Results The concentration of Zn and other micronutrients (Mn, Fe, and Cu) was higher in grains treated with foliar Zn during grain-filling (early milk/dough) than those treated at stem elongation. The increase in Zn concentration of wheat grain with foliar application during grain-filling can be attributed to the intense localization of Zn in the aleurone layer, modified aleurone, crease tissue, vascular bundle, and endosperm cavity, and to a modest localization in endosperm, which is the most dominant grain tissue. These tissues and the Zn they contain are presumed to remain after milling and can potentially increase the Zn concentration in wheat flour.
Conclusions By using XFM, it was shown that foliar Zn spray represents an important agronomic tool for a substantial Zn enrichment of different fractions of wheat grain, especially the endosperm. Further investigation of the chemical speciation of Zn in the endosperm is recommended to assess Zn bioavailability in harvested whole grain of wheat that has been biofortified through different timing of foliar Zn application.

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Keywords Biofortification · Foliar application · Wheat grain · Zn localization · Zn fertilizer · X-ray fluorescence microscopy · Aleurone layer · Endosperm · Endosperm cavity

Abbreviations

XFM	X-ray fluorescence microscopy
ICP-OES	Inductively coupled plasma optical emission spectroscopy
LA-ICP-MS	Laser ablation inductively coupled plasma mass spectroscopy
SIMS	Secondary ion mass spectroscopy
PIXE	Photon induced X-ray emission
XANES	X-ray absorption near edge structure spectroscopy

Introduction

According to World Health Organization estimates, more than 1 billion people suffer from malnutrition caused by deficiency of micronutrients, such as, Co, Fe, I, Se, and Zn (WHO 2002; de Benoist et al. 2007a, b, c; de Benoist and Delange 2007; Welch 2008). Zinc deficiency can cause severe health complications, such as impairments in immune system, physical development, and brain function, especially in children (Hotz and Brown 2004). For example, immune system impairment can exacerbate the incidence of infectious disease such as diarrhoea and pneumonia (Gibson et al. 2008). Following from the identification of Zn deficiency as a top priority area in the 2008 Copenhagen Consensus (The Copenhagen Consensus Conference 2008), therapeutic Zn supplementation is now being promoted with oral rehydration therapy (ORT) in developing countries to combat diarrhoea in children (Horton et al. 2009). High consumption of cereal-based foods that are inherently low in Zn and other micronutrients is very common in countries where high Zn deficiency has been documented (Bouis and Welch 2010; Cakmak 2008; Gibson et al. 2008). Therefore, biofortification of cereals with Zn could serve as a nutritional intervention in these countries. Biofortification of cereal grains with Zn and determining the bioavailability of stored Zn compounds in edible part of the grain have been identified as high-priority research areas in plant nutrition (Cakmak 2002; Prasad et al. 2014). Currently, genetic and agronomic approaches have been identified as biofortification options to increase the accumulation of Zn in grains. However, due to the nature of plant breeding as a long-term process, agronomic biofortification seems preferable as an immediate short-term solution.

Accumulation of Zn in the grain may be limited by barriers to Zn transport, for example, from the rachis into the vascular bundle due to xylem discontinuity in the maternal tissue of wheat grain (O'Brien et al. 1985; Zee and O'Brien 1970). This transport barrier may reduce the efficiency of soil-applied Zn fertilizer, in addition to other soil chemical and physical factors that affect Zn fertilizer availability such as high pH, low organic matter, and soil moisture (Alloway 2004; Barrow 1993; Fageria 2009). However, given the relatively high phloem mobility of Zn in cereals (Marschner 1995) and the fact that peak Zn accumulation occurs during the early milk stage of wheat (Ozturk et al. 2006), foliar Zn application during the grain-filling stage could potentially increase Zn translocation into grains during the grain-filling period. Several experiments have been carried out in the past investigating impact of timing of foliar Zn spray on accumulation and localization of Zn in wheat grain by using dye staining, ICP-OES and laser ablation inductively coupled plasma mass spectroscopy (LA-ICP-MS) (Cakmak et al. 2010b; Kutman et al. 2011; Ozturk et al. 2006; Zhang et al. 2012).

The advantages and disadvantages of various techniques have been recently discussed by Zhao et al. (2014). The dye staining technique provides rapid visualization of Zn distribution in wheat grain based on the complexation reaction between Zn and dithizone (1,5′-diphenyl thiocarbazone) and has been used to differentiate Zn distribution among the aleurone, endosperm, and embryo in wheat and rice (Cakmak et al. 2010b; Choi et al. 2007; Ozturk et al. 2006). The LA-ICP-MS method involved sample fixation and embedding in resin prior to scanning along the transverse section of embedded grain to determine Zn distribution (Cakmak et al. 2010a). While the LA-ICP-MS technique provides better information on Zn concentrations in the sampled region than that obtainable with the dye technique, it suffers from coarse spatial resolution in comparison to that required to visualize the complexity of Zn localization in the grain.

Understanding the complexity of micronutrient distribution, especially at tissue and cellular level, requires specialized techniques such as X-ray fluorescence microscopy (XFM) (Lombi et al. 2009, 2011b, c; Kyriacou et al. 2014) and secondary ion mass spectroscopy with

nanometer resolution (nanoSIMS) (Moore et al. 2010, 2012a, b). Although XFM is a well-known imaging technique, it has not been used to image metal distribution of large areas of hydrated biological samples until quite recently (Lombi et al. 2011a). The recent advance in high resolution XFM with fast detectors has now opened opportunities for mapping Zn distribution in whole transverse sections of cereal grains in great detail and with massively improved time efficiency (Lombi et al. 2011a, c; Ryan et al. 2010a, b). The XFM technique also has the capability to simultaneously map the distribution of other Zn-associated micronutrients.

The objective of the present study was to investigate, using high definition and micron-resolution XFM, the localization of Zn and associated micronutrients in wheat grain biofortified with Zn under field conditions by foliar spray of Zn at different growth stages. Transverse sections of wheat grains biofortified via foliar Zn application were imaged at the Australian Synchrotron. The high definition XFM elucidated Zn localization in wheat grains that can be used to improve biofortification efforts and to optimize grain processing. To our knowledge, this work is the first example of synchrotron XFM being used to study localization of Zn in wheat grains, which were biofortified through different timing of foliar Zn applications under field conditions.

Materials and methods

Field trials with foliar Zn application

Field trials were carried out at two locations in Turkey; Adana, and Samsun, to study the role of foliar Zn applications at different growth stages of bread wheat (*Triticum aestivum* L) in increasing grain Zn concentrations. The detail of the field trials have been described by Cakmak et al. (2010a). Briefly, foliar $ZnSO_4 \cdot 7H_2O$ (0.5 % *w/v*) was applied at the rate of 4 kg ha^{-1} to wheat plants at different stages: i) stem elongation/booting, ii) early milk/early dough, and iii) a combination of i) and ii). A treatment without foliar Zn application served as a control. The soils at the two locations had adequate Cu, Fe, Mn and Zn based on soil test (DTPA extraction) and fertilizer application guidelines for the area. Nitrogen and P were applied at the rates of 80 kg N ha^{-1} (as ammonium nitrate) and 80 kg P_2O_5 ha^{-1} (as triple superphosphate), respectively. Harvested grains from each treatment was analysed for Zn, Fe, Cu and Mn by ICP-OES (Vista-Pro Axial; Varian Pty Ltd., Mulgrave, Australia) following a microwave chamber (MarsExpress CEM Corp., Matthews, NC) digestion with concentrated HNO_3 and H_2O_2. The ICP-OES data were checked against certified values of a reference material (SRM 8436 Durum Wheat Flour, National Institute of Standards and Technology, Gaithersburg, MD). Analysis of variance (ANOVA) in the data from each location was carried out using a generalized linear model and a protected Fisher's LSD was used for mean comparisons. All the statistical analyses were carried using XLSTAT statistical analysis add-in for Microsoft Excel (Addinsoft, New York, NY). Selected grains from all the treatments from both locations were then prepared for X-ray fluorescence microscopy (XFM).

Sample preparation for X-ray fluorescence microscopy (XFM)

Thin transverse sections (−50 μm) from the middle part of selected wheat grains from each of the treatments described above were prepared using a Leica VT1000 S vibrating blade microtome "vibratome" (Leica Microsytems, Wetzlar) as described by Lombi et al. (2011c). The proximal end of the grains was fixed on a plastic base with an adhesive. Progressive thin sections were removed from the distal end of the grains using the vibratome, with care being taken to ensure a flat surface. On getting to the desired layer of the grain, the sticky part of a polyimide film (Kapton) was carefully taped on the flat surface with −1 and −10 mm sticking over the top and bottom parts of the grain cross-section, respectively. The transverse section was then cut and captured on the Kapton film, with care being taken to retain all the features. This sample preparation method allowed for sectioning of the grains directly on a contamination-free polyimide tape without the need for embedding, which is ideal for XFM imaging (Donner et al. 2012; Lombi et al. 2009; Meharg et al. 2008).

Microphotographs of the thin sections were obtained using a Leica DM E compound microscope fitted with a Leica MPS photo system (Leica Microsystems, Wetzlar). With this protocol, morphological features of the grains such as; seed coat, aleurone layer, crease

tissue, vascular bundle, endosperm cavity, endosperm and layer were retained with high fidelity.

X-ray fluorescence microscopy (XFM) experiment

The imaging experiment was carried out at the X-ray Fluorescence Microscopy (XFM) beamline of the Australian Synchrotron (Paterson et al. 2011). The beamline uses an in-vacuum undulator to produce a brilliant X-ray beam over the 4–25 keV energy range. From this beam, a double Si (111) crystal monochromator then selects a narrow range of X-ray with a mean energy of 12.7 keV. A Kirkpatrick-Baez mirror pair focused the monochromatized beam to a spot size of approximately 2 μm^2 and the grain thin sections were scanned through this focus. Prior to mounting the grain thin sections on the sample stage, they were first mounted on a polyethylene transparency film (140×100 mm) with three pre-cut rectangular grids (10×100 mm). Mounting was done first by trimming the Kapton tapes on both right and left sides of the transverse section to −1 mm. The bottom part of the Kapton tape holding the cross-section was then taped along the edge of the grid, such that all the sections were aligned side-by-side along the rectangular grid.

A Maia 384 array detector was used to collect the X-ray fluorescence signal during the experiment (Ryan et al. 2010b). The annular configuration of the Maia 384 detector allowed for the collection of the emitted fluorescence from the sample in a backscatter geometry. The detector face was within about 1 mm of the sections, and the detector chip about 10 mm. The Maia detector system operates in event mode and as such does not record spectra; rather, photon event energies are streamed to disk along with stage encoded position as the scan progresses continuously (or 'on the fly') through the beam in an x-y raster pattern. Setting the stage to a maximum velocity of 8.2 mm s^{-1} resulted in a pixel (2 μm) transit time of 0.244 ms. It took just 3.5 h to collect very high definition multi-element maps of an area −268 mm^2, a feat that has not been achieved in XFM imaging until recently (Lombi et al. 2011c).

XFM analysis

The XFM data was processed using the GeoPIXE software (Ryan 2000; Ryan et al. 2012). This software incorporates a Dynamic Analysis (DA) matrix transform method, originally designed for photon induced X-ray emission (PIXE) imaging but now adapted to SXRF imaging (Ryan et al. 2005). The DA matrix deconvolutes the fluorescence events to provide quantitative images of projected elemental content. Calibration of the DA matrix was carried out using a multi-element foil (MEF) containing Cr, Fe, Ni and Ti (James et al. 2011) after correcting for self-absorption in the sample, absorption in air, geometric effect and the efficiency response of the detector. The measured X-ray signal in each pixel was related to the modelled X-ray yield of the grains cross-sections with an assumed tissue composition being 50 μm of cellulose ($C_6H_{10}O_5$)n). This assumption is only used to apply self-absorption corrections (small in this system) and so results in negligible error. Uneven sample thickness encountered in some cross-sections (likely due to resonances in the vibratome) was corrected by normalizing the fluorescence yield to Compton scattering in GeoPIXE. Semi-quantitative elemental maps of Cu, Fe, Mn, and Zn were thus extracted from the megapixel image strip to compare the concentration (as areal density) of these micronutrients in biofortified wheat grains. Semi-quantitative maps were presented in this study because of the strong dependence of absolute concentration on the thickness and density of grain thin sections, which were extremely difficult to control. It is extremely useful to acquire all specimens with one scan as done in this experiment, as this completely rules out the possibility of artefacts resulting from changes in experimental conditions between scans.

Results

Concentration of micronutrients in whole grains

Foliar Zn application increased grain Zn concentration at both locations (Table 1). At Adana, foliar application during the late season was the most effective in increasing grain Zn, which was not different than double foliar Zn application during early and late season. At Samsun, however, double foliar Zn application during early and late season was the most effective in increasing grain Zn. Foliar Zn application apparently increased grain Fe at Samsun, but there was no effect of foliar Zn on grain Fe at Adana and on other micronutrients (Cu and Mn) at both locations.

Table 1 Concentrations of Zn, Fe, Mn and Cu in whole grain of bread wheat (*Triticum aestivum* L) treated with foliar Zn at different growth stages

Foliar Zn application stage	Adana				Samsun			
	Zn[a] mg kg^{-1}	Fe[a]	Cu	Mn	Zn[a] mg kg^{-1}	Fe[a]	Cu	Mn
No foliar Zn (control)	32a	36	5.0	40	23a	29a	4.7	25
Stem elongation/booting	51b	39	5.1	42	42b	35bc	4.9	25
Early milk/dough	57bc	35	4.6	42	44b	33b	4.8	25
Stem elongation/booting+early milk/boot	65c	36	4.9	40	56c	36c	5.1	27

Values shown are means of 4 replicates. Means with same letter are similar at 5 % significance level while means in columns with no letter are not significantly different

[a] As reported by Cakmak et al. (2010a).

Identification of morphological features in grain transverse sections

The microphotograph of a selected transverse section used for XFM imaging shows that relevant morphological features such the crease tissue, endosperm cavity and vascular bundle, endosperm and aleurone layer were retained (Fig. 1).

Zinc localization

The localization of Zn in grains with or without foliar Zn application at the Adana and Samsun locations are shown in Fig. 2. Without foliar application, Zn was mostly concentrated in the vascular bundle and modified aleurone layer, and to a lesser extent in the crease tissue and aleurone layer. However, Zn was inherently low in the endosperm (Fig. 2a and e). Single foliar Zn application early in the growth stages (stem elongation/booting) appeared to have increased the Zn concentration in the aleurone layer but not so much in the crease tissue. Single foliar Zn application later in the growth stages and double foliar Zn application at both earlier and later in the growth stages markedly increased the concentration of Zn in all the tissues as well as in the endosperm (Fig. 2c, d, g and h). The multiple layers of aleurone in the dorsal part of the grain in Fig. 2g (as well as in Figs. 3g, 4g, and 5g) are due to a thicker and denser section of this part of the grain. Compton normalization can remove the issues of thickness in relation to elemental concentration, however, the multiple aleurone cell layers still appear in the two dimensional images of the volume analysed due to the penetrating nature of X-rays.

Localization of other micronutrients

Manganese in the grains from the control treatment was mainly concentrated in the vascular bundle in the grain

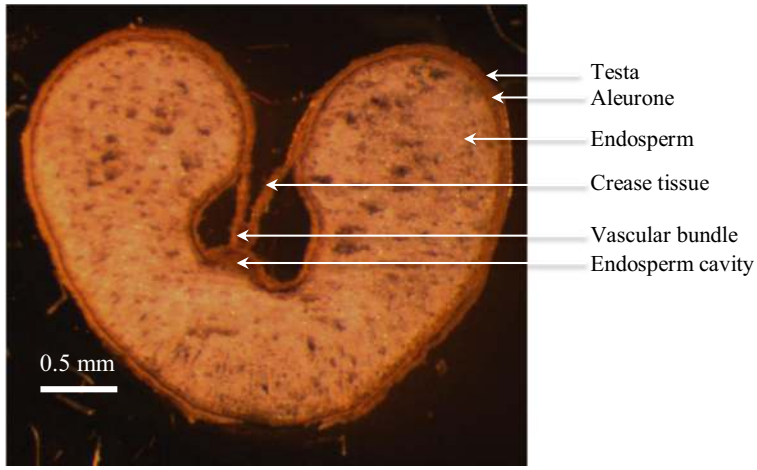

Fig. 1 A microphotograph of a transverse section of a selected wheat grain (same as shown in panel d of Figs. 2, 3, 4 and 5) showing relevant morphological features

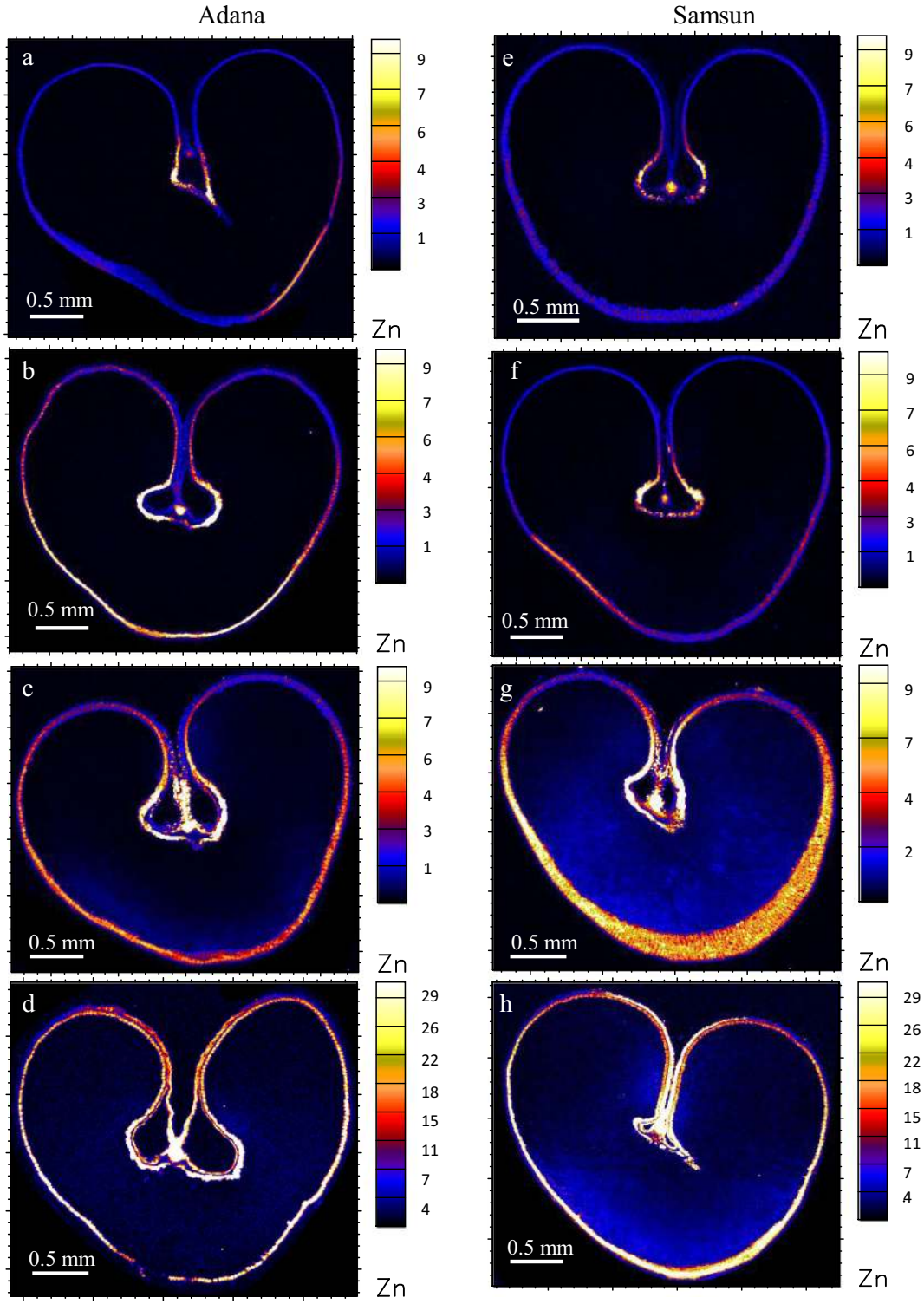

Fig. 2 Localization of Zn (ng cm^{-2}) in wheat transverse sections from Adana with: **a** no foliar Zn, **b** single foliar application at stem elongation/booting, **c** single foliar application at early milk/early dough, and **d** a combination of foliar application at stem elongation/booting and early milk/early dough; and from Samsun with: **e** no foliar Zn, **f** single foliar application at stem elongation/booting, **g** single foliar application at early milk/early dough, and **h** a combination of foliar application at stem elongation/booting and early milk/early dough

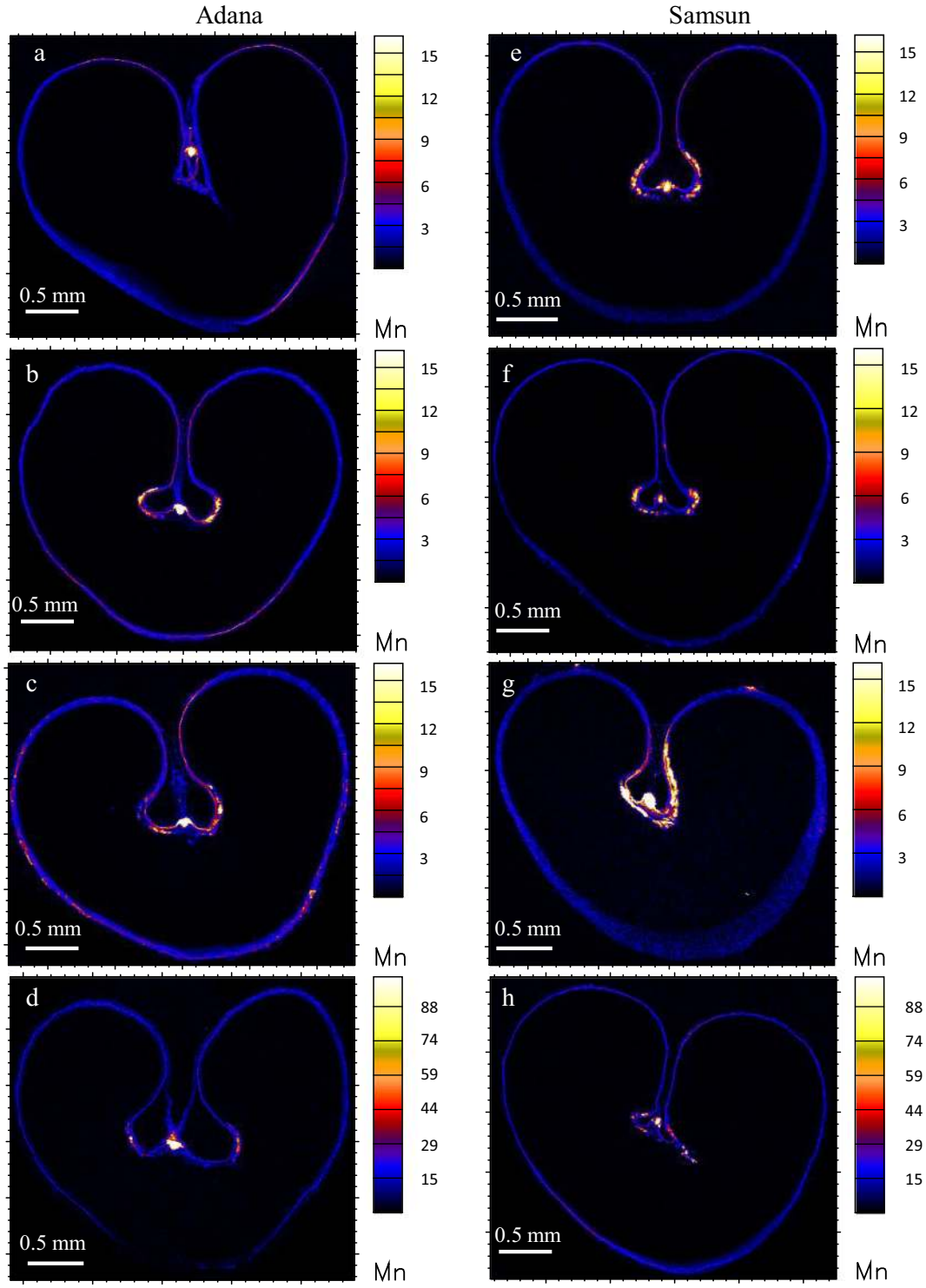

Fig. 3 Localization of Mn (ng cm^{-2}) in wheat transverse sections from Adana with: **a** no foliar Zn, **b** single foliar application at stem elongation/booting, **c** single foliar application at early milk/early dough, and **d** a combination of foliar application at stem elongation/booting and early milk/early dough; and from Samsun with: **e** no foliar Zn, **f** single foliar application at stem elongation/booting, **g** single foliar application at early milk/early dough, and **h** a combination of foliar application at stem elongation/booting and early milk/early dough

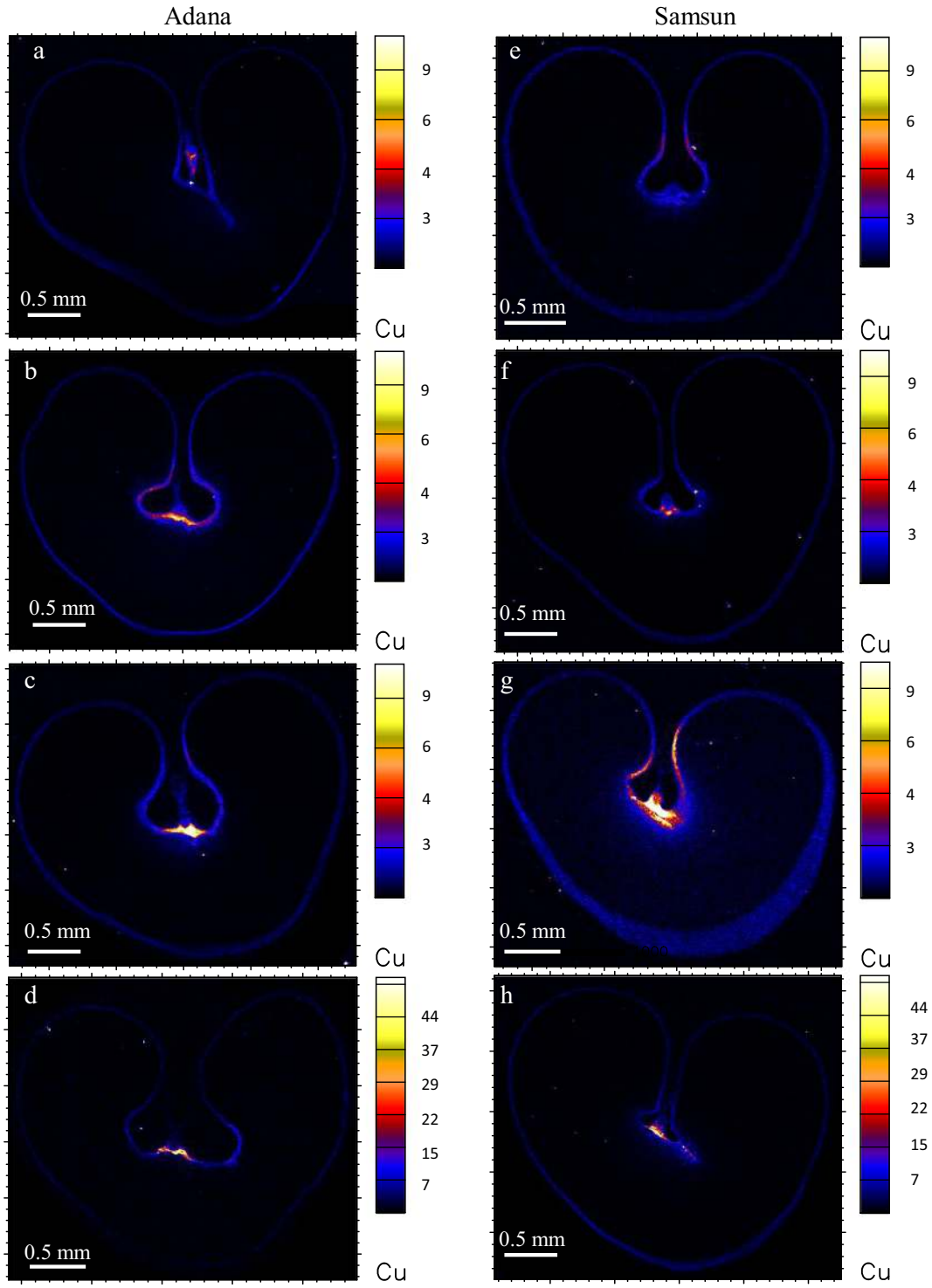

Fig. 4 Localization of Cu (ng cm^{-2}) in wheat transverse sections from Adana with: **a** no foliar Zn, **b** single foliar application at stem elongation/booting, **c** single foliar application at early milk/early dough, and **d** a combination of foliar application at stem elongation/booting and early milk/early dough; and from Samsun with: **e** no foliar Zn, **f** single foliar application at stem elongation/booting, **g** single foliar application at early milk/early dough, and **h** a combination of foliar application at stem elongation/booting and early milk/early dough

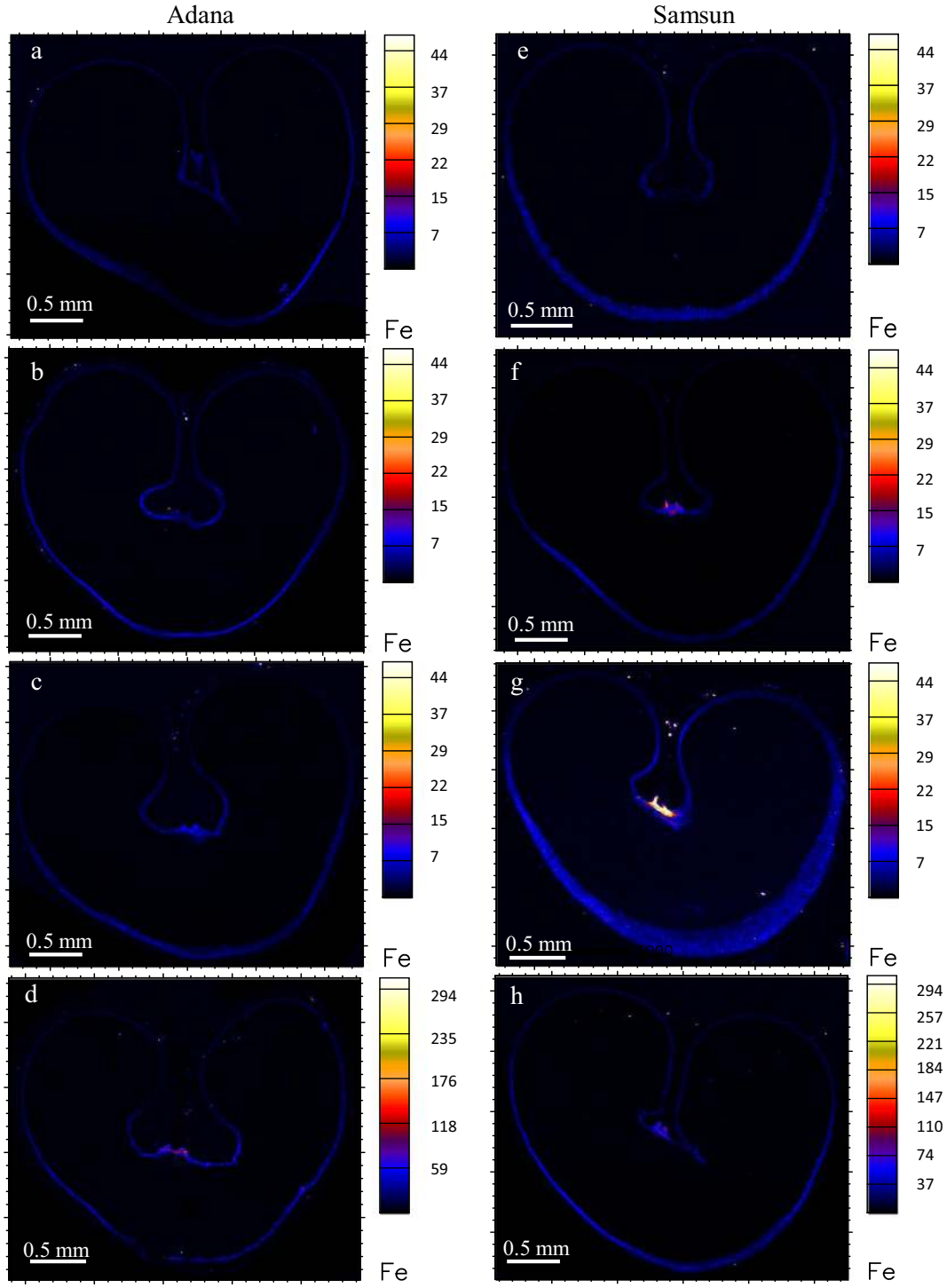

Fig. 5 Localization of Fe (ng cm^{-2}) in wheat transverse sections from Adana with: **a** no foliar Zn, **b** single foliar application at stem elongation/booting, **c** single foliar application at early milk/early dough, and **d** a combination of foliar application at stem elongation/booting and early milk/early dough; and from Samsun with: **e** no foliar Zn, **f** single foliar application at stem elongation/booting, **g** single foliar application at early milk/early dough, and **h** a combination of foliar application at stem elongation/booting and early milk/early dough

from Adana and in both vascular bundle and modified aleurone layer in the grain from Samsun. Similar to Zn, the aleurone layer and crease tissue contained a lower concentration of Mn than the vascular bundle, which was similar across the treatments, from the control to the late season foliar Zn application. It is interesting to note a marked increase in Mn concentration in the aleurone layer with double foliar Zn application at both early and late growth stages (Fig. 3d and h, note changes in scale). This higher Mn concentration at the late growth stage may be due to co-translocation of Mn with Zn, as no additional Mn was added to these treatments.

In some respects, the localization of Cu differed markedly from that of Mn and Zn. Copper was mainly localized in the endosperm cavity between the vascular bundle and the modified aleurone layer, and it seemed to increase with the foliar Zn application treatments; from a single early foliar application to double early and late foliar applications (Fig. 4). However, similar to the distribution of Mn, there was a marked increase in the distribution of Cu in the aleurone layer with a combination of early and late foliar Zn applications (Fig. 4d and h).

Iron localization and concentration aleurone later was unchanged across the treatments, with the exception of treatments with double foliar Zn application early and later in the season, which had a much higher Fe concentration in the aleurone layer and endosperm cavity (Fig. 5). Distribution of Fe has been observed to be confined to the aleurone layer in other studies and this was attributed to a likely complexation of Fe with phytate in the protein storage vacuole of the aleurone (Becraft 2007; Persson et al. 2009). The Fe "hot spot" in the nucellar projections of grain from Samsun with a single late foliar Zn application (Fig. 5g) may be due to sectioning artefact or dust contamination.

Discussion

Zinc localization in Zn-biofortified wheat grains

The XFM images in the current study, although semi-quantitative, provide information on the localization of Zn in more detail than those from LA-ICP-MS line scans of the transverse sections previously reported by Cakmak et al. (2010a). While those authors had reported an increase in Zn concentration along transects of the transverse section of the distal end of the grain with later timing of foliar Zn application, the present result shows the exact location and average concentration of Zn in different tissues of the whole transverse section. Increased Zn in the endosperm, while less than Zn increases in other tissues, suggests that foliar Zn application during grain filling may have overcome some of the transport barrier from the rachis into the endosperm. Although the increase in Zn concentration in the endosperm was much lower than that in the modified aleurone, crease tissue, and vascular bundle, the total amount of Zn transported into endosperm should be much higher than other seed parts since the endosperm part accounts for about 80 % of the grain weight (Stomph et al. 2011). The increase in endosperm-Zn might be of great importance regarding the human nutrition. Endosperm part of wheat is processed into white flour and represents the most commonly eaten part of wheat. Therefore, any significant change in endosperm Zn would contribute markedly to human Zn nutrition. In addition, phytate that is known as a compound limiting Zn bioavailability to humans is very low and even not measurable in wheat grain endosperm (Velu et al. 2014). Therefore, it can be speculated that the Zn increase in the endosperm of wheat by foliar Zn spray might be largely bioavailable because of the very low amounts of phytate in this grain part. These results indicate that increasing the pool of Zn in vegetative tissue during the seed filling stage through foliar application of Zn fertilizers represents an important agronomic tool to achieve particular increases in Zn both in whole grain and in endosperm. The likely pathways of Zn transport into the endosperm are discussed further in the following section.

Co-localization of zinc with other micronutrients

The localization of Cu, Mn, and Zn in transverse sections of grain without foliar Zn application (control) and that with foliar Zn application at early milk/early dough is shown as a tri-color RGB map (Fig. 6). In the control grain, Cu and Zn are co-localized in the aleurone layer while Mn was localized in the testa. The presence of Mn and Zn in high concentration in the aleurone layer has been reported previously (Mazzolini et al. 1985; Stomph et al. 2011), although at significantly lower lateral image resolution than in the current study. The extension of Mn outside the aleurone layer into the seed coat has been reported also for barley and rice using XFM (Lombi et al. 2011c; Kyriacou et al. 2014). It appears the accumulation of Mn along the seed coat is common to cereals.

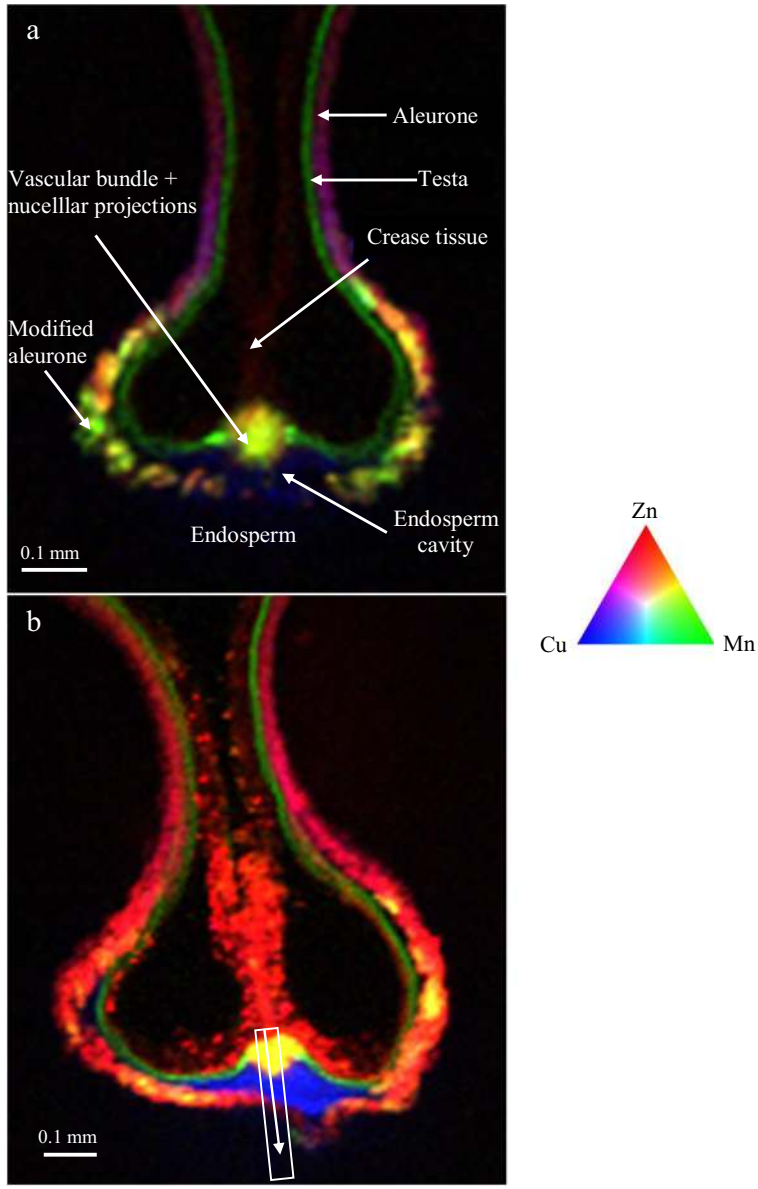

Fig. 6 Tricolor RGB maps showing the co-localization of Cu (*blue*), Mn (*green*), and Zn (*red*), in the transverse sections of grain with (**a**) no Zn foliar application (in Fig. 2e) and (**b**) with foliar application at early milk/early dough (in Fig. 2c) to show possible transport pathway of Zn from the crease tissue into the endosperm. Overlap between Mn (*green*) and Zn (*red*) is shown as yellow

Possible transport pathway of zinc into the endosperm

Elemental traverses along the sagittal plane of Fig. 6b provide some insights into transport of Zn into the endosperm (Fig. 7). The increasing concentration of Zn in the crease tissue with foliar Zn application warrants closer attention, as some studies have suggested that Zn is distributed within grains through the crease phloem (Cakmak et al. 2010a; Pearson et al. 1998; Wang et al. 2011). Xylem-to-phloem transfer within the palea has also been reported to be significant in transporting Zn into the grain after blocking phloem transport in the rachilla by heat girdling (Pearson et al. 1995). With foliar Zn application during grain-filling, it is most likely that Zn accumulates in the posterior part of palea and lemma as well as along the crease. This may explain the highest concentration of Zn in the crease with foliar Zn application later during grain-filling. Given that xylem-to-phloem transfer occurs in the palea (close to the floral axis) and that solutes are unloaded from the phloem into the apoplast at the site of xylem discontinuity in the floral axis (Jenner 1985a, b, c),

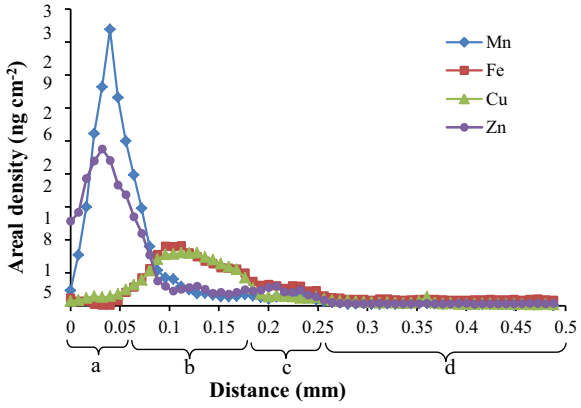

Fig. 7 Traverse projections of the rectangle in Fig. 6b showing the concentration profile (ng cm^{-2}) of Cu, Mn and Fe from the vascular bundle (**a**) across the endosperm cavity (**b**) and modified aleurone (**c**) into the endosperm (**d**)

foliar-applied Zn that accumulated at the floral axis may have penetrated into the apoplast and subsequently transported into the grain via the phloem of the vascular bundle. This may explain the higher Zn concentration in the vascular bundle with foliar Zn application during grain-filling.

Another interesting observation is the high concentration of Cu and Fe in the endosperm cavity (Fig. 7). This suggests that Cu and Fe transport from maternal to filial tissue are similarly regulated and occurs at a different timing than Zn and Mn transport.

Although the physiological processes controlling the distribution of Zn and other micronutrient in cereal grains are very complex as pointed out by several authors (Borg et al. 2009; Cakmak et al. 2010b; Olsen and Palmgren 2014; Palmgren et al. 2008; Tauris et al. 2009), evidence from functional morphology and ultrastructural studies suggests that assimilates are generally unloaded from the vascular bundle and then transported across the transfer cells of the nucellar projection into the endosperm cavity (Borg et al. 2009; Frazier and Appalanaidu 1965; Krishnan and Dayanandan 2003; Oparka and Gates 1981a, b; Thorne 1985). From the endosperm cavity, the cells of the modified aleurone layer transport these assimilates into the aleurone layer, the starchy endosperm and the embryo. Thus, the Zn concentration gradient from the vascular bundle, across the nucellar projection and modified aleurone into the endosperm along the sagittal plane suggests vascular bundle-endosperm cavity-modified aleurone as a possible Zn transport pathway into the endosperm following foliar Zn application. Clearly, there is a transport barrier from the vascular bundle into the endosperm. Wang et al. (2011) reported a limited transfer of Zn into the endosperm using enriched stable ^{70}Zn labelling and LA-ICP-MS. A large gradient in the concentrations of Fe, Mn, and Zn between the crease and endosperm of wheat grain grown under adequate Zn fertilization (5 kg ha^{-1}) has also been reported by (Stomph et al. 2011) in investigating Zn transport limitations in developing grains.

Implication of zinc localization on grain processing

During the milling of wheat grain to make flour, the seed coat and embryo and possibly the aleurone layer are often removed (Welch and Graham 1999). Our results suggest that even when the seed coat and the aleurone layer are removed, significant amount of Zn may remain in the crease tissue, modified aleurone layer and vascular bundle. The contribution of the Zn in these tissues to the overall Zn concentration in biofortified grain could be substantial given that they stretch along the full longitudinal section of the wheat grain.

Future studies using XFM

Understanding metal transport from the maternal to filial tissue of grains has been limited due to a lack of appropriate methods for studying the localization of these metals at tissue and cellular levels. Clearly the XFM technique described in our experiments offers this capability. XFM can also be used to investigate the speciation of Zn in biofortified wheat grain, as this is important for bioavailability of dietary Zn. A recent report on the association of Zn with the globoid of protein storage vacuole in the aleurone layer of wheat is changing the widely held belief of Zn storage as Zn phytate in the grain (Regvar et al. 2011). A further study is therefore required to investigate the Zn speciation in biofortified grain using spatially-resolved X-ray absorption near edge structure (μ-XANES), especially in the endosperm.

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