Steeping and germination of wheat (*Triticum aestivum* L.). II. Changes in spatial distribution and speciation of iron and zinc elements using pearling, synchrotron X-ray fluorescence microscopy mapping and X-ray absorption near-edge structure imaging

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List of abbreviations

Germinated pearling	GP
NA	nicotianamine
Ungerminated pearling	UGP
XANES	X-ray absorption near-edge structure
XRF	X-ray fluorescence

ABSTRACT

Iron (Fe) and zinc (Zn) in wheat are mainly present as insoluble phytates in the aleurone cells. Phytate breakdown during steeping and germination of wheat enhances their bio-accessibility. However, the accompanying redistribution within the grain and changes in speciation are still unclear. We unravelled these changes using a combination of wheat fractionation by pearling, X-ray fluorescence microscopy mapping and X-ray absorption near-edge structure imaging. A significant fraction of Zn and Fe migrates to radicles and coleoptiles during germination. The remainder has low water extractability (≤ 8%). The high water extractability (26-27%) of Zn in the radicle and coleoptile indicates its mobility in the seedling. Zn migrates from the aleurone to the pericarp and embryonic tissues, while Fe is dominantly present in the scutellum tissue attached to the seedling. The co-localisation of Zn and sulfur (S) in the developing embryo suggests that translocated Zn is bound to S-containing peptides. In aleurone cells Fe remains phytate bound and although Zn is phytate and S-bound, slight changes in its speciation occur during germination. This study partially explains the impact of steeping and germination on mineral bio-accessibility and opens possibilities for enhancing nutritional quality during food processing.

1. INTRODUCTION

From the outside to the inside, a wheat grain consists of pericarp, seed coat, nucellar epidermis, aleurone and starchy endosperm. The embryo is located on the dorsal side and the longitudinal crease runs over the length of the ventral side of the grain (Delcour and Hoseney, 2010). As a staple food, wheat is the predominant source of human dietary carbohydrate in Western countries where it contributes to about 25% of the daily caloric intake (Cakmak, 2008). Moreover, it also contains significant levels of protein, dietary fibre and micronutrients such as vitamins, mineral elements and phytochemicals (Delcour and Hoseney, 2010). Cereal products are responsible for about 40 and 25% of the average daily intake of iron (Fe) and zinc (Zn) in adults (aged 19–64) in the UK, respectively (Bates et al., 2014).

About 85% of wheat phosphorus (P) is stored as phytic acid, which occurs as granules embedded in protein-rich globoid structures in the aleurone and chelates divalent cations such as Fe, Zn, calcium (Ca), manganese (Mn), magnesium (Mg) and copper (Cu) (Schlemmer et al., 2009). This phytate chelation causes Fe and Zn to be only 3-5% bio-accessible (Lemmens et al., 2018).

The selective mechanism by which plants accumulate mineral nutrients is tightly controlled. During grain development, nutrients are transported from leaves and stem via phloem transport to the crease region (White, 2012). The mineral elements are transferred through the crease vascular parenchyma cells and pigment strand to the nucellar projection. From there, they are transported through transfer cells in the vascular bundle into the endosperm cavity. During grain maturation, they are distributed from the modified aleurone to the aleurone (± 70%), the starchy endosperm (± 20%) and the embryo (± 10%) to be stored as nutritional reserve for future embryonal growth (Eastwood and Laidman, 1971; Ozturk et al., 2006; Tauris et al., 2009). The portion of specific elements transported and accumulated during maturation depends on their mobility.

De Brier et al. (2015; 2016) revealed that the highest concentrations of Mn are present in the seed coat and those of Ca in the outermost bran layers (mainly pericarp) of the wheat grain. The starchy endosperm also contains some Ca (De Brier, Gomand, et al., 2015). Ca and Mn have low mobility in the phloem and may enter the grain through a direct xylem supply of the pericarp (Pearson et al., 1999; White, 2012). Mg, Cu, Fe and Zn are mostly confined to the aleurone, while significant amounts of potassium (K) occur in both the aleurone and pericarp. Zn is to a minor extent also present in the starchy endosperm where it mainly occurs as a soluble complex with nicotianamine (NA) and/or bound by thiol groups of cysteine-containing peptides (Eagling et al., 2014). Zn and Fe have good and moderate phloem mobility, respectively (Marschner, 2012). X-ray absorption near-edge structure (XANES) spectroscopic imaging confirmed that Fe in the aleurone cells mainly occurs as phytate Fe^{2+} (De Brier, Gomand, et al., 2015; Neal et al., 2013). The embryo contains relatively high levels of Mn, Zn and Cu, while Fe and P are mainly concentrated in the scutellum (De Brier et al., 2016). Mn and Cu elements in the embryo support seedling growth during grain germination (Takahashi et al., 2009). Zn is a critical micronutrient. It is important during seedling development [*e.g.* cofactor plant hormones, protein synthesis, cell elongation (Cakmak et al., 2010)]. More than 300 enzymes require Zn which contributes to structural integrity, biological function and organism stress tolerance (Marschner, 2012). In the protein storage vacuoles of the embryo tissue, Zn is mainly bound to sulfur (S) containing peptides such as metallo-thionein (Persson et al., 2009).

The mobilisation of many nutrient elements during germination is correlated with the activation and synthesis of phytase (White, 2012). Phytase hydrolyses ester bonds in phytic acid, thereby releasing phosphate, myo-inositol and mineral elements (Schlemmer et al., 2009). As a consequence of wheat germination (15 °C for 5 days), Fe and Zn bio-accessibilities increase from 5 and 3% to 14 and 15%, respectively (Lemmens et al., 2018). The released Zn especially accumulate in the meristematic tissues of the embryo as shown with dithizone staining (Ozturk et al., 2006). Takahashi et al. (2009) showed with X-ray fluorescence (XRF) microscopy (10-50

μm resolution) that during 24-36 h of rice grain germination, Fe is transported from the scutellum to the seedling where it is involved in respiration, photosynthesis and nitrogen fixation (Hell and Stephan, 2003). In addition, Lemmens et al. (2018) found that ferrous Fe in wheat grains is oxidised during steeping and germination and that Fe is no longer only bound to phytate structures.

The present work was set up to examine migration and changes in chemical speciation of mineral elements during wheat germination. This was done using high-resolution μ-XRF microscopy and XANES spectroscopic imaging, respectively. We studied the impact of different germination conditions on the mineral distribution in nutrient-dense regions such as the aleurone, crease, coleoptile and radicle. The gathered knowledge contributes to a better understanding of the physiological processes behind Fe and Zn transport and accumulation during germination of cereal grains. Moreover, these findings shed light on the changes in mineral bio-accessibility as a result of wheat germination.

2. MATERIALS AND METHODS

2.1 Materials

Cellule winter wheat was kindly supplied by Limagrain (Avelgem, Belgium). A reference wheat (NIST1567) flour sample with certified element composition was obtained from the National Institute of Standards and Technology (Gaithersburg, MD, USA). All chemicals and reagents were of analytical grade and purchased from Sigma-Aldrich (Bornem, Belgium).

Wheat steeping and germination procedures are described in Lemmens et al. (submitted). Sample A was prepared by steeping at 15 °C for 29 h, germinating at 15 °C for 120 h, flash-freezing with liquid N_2 and freeze-drying. Sample B was prepared similarly but by steeping at 15 °C for 36 h and germinating at 26 °C for 120 h, which showed optimal phytate and cell wall hydrolysis (Lemmens et al., submitted).

2.2 Pearling of (un)germinated wheat grains

To investigate the translocation of Zn and Fe and changes in their co-localisation with P and S in different wheat tissues upon germination, wheat grains were pearled. Ungerminated wheat grains and germinated (sample A) wheat grains were abraded after removing radicle and coleoptile using a pearling device type TM05 (Satake, Bredbury, UK) to remove and collect in a successive approach 5.0, 10.0, 15.0 and 25.0% of the grain weight. The ungerminated pearling fraction (UGP) or germinated pearling fraction (GP) obtained in each step are further referred to as pearlings 0-5% [(U)GP0-5%], pearlings 5-10% [(U)GP5-10%], pearlings 10-15% [(U)GP10-15%] and pearlings 15-25% [(U)GP15-25%]. The pearled grains were also collected.

The endogenous phytase of the pearled samples was inactivated by suspending an aliquot (0.50 g) in 80% ethanol (10.0 ml) and evaporating the added ethanol at 95 °C.

The mineral extraction process involved suspending the phytase inactivated samples in 10.0 ml Milli-Q water (18.2 MΩ cm at 25 °C, Milli-Q Plus, Merck Millipore, Darmstadt, Germany),

mixing [30 minutes (min), 150 rotations per minute (rpm), room temperature], centrifugation (10 min, 5,500 g, room temperature) and filtration (MN615, 4-12 µm, Macherey-Nagel, Düren, Germany) to obtain the aqueous extracts.

Samples (0.05 g) were prepared for elemental analysis by digesting with 1.0 ml ultrapure 25% *v/v* HNO_3 and subsequently with 2.0 ml *aqua regia* (75% *v/v* ultrapure HCl and 25% *v/v* ultrapure HNO_3) at 150 °C, followed by cooling and dilution with Milli-Q water to 10.0 ml.

The S content of the samples (pearling fractions, pearled grains and extracts) was determined with inductive coupled plasma optical emission spectrometry (iCAP 7400 ICP-OES Analyser, Thermo Fisher Scientific, Waltham, MA, USA). The Zn, Fe and P contents were determined with inductive coupled plasma mass spectrometry (Agilent 7700x ICP-MS, Santa Clara, CA, USA).

The reported elements were recovered within 10% of the certified values of the NIST1567 wheat reference (data not shown). The percentage of mineral elements extracted was calculated as follows:

$$Extractability\ (\%) = \frac{mass\ of\ element\ in\ extract\ (g\ dm)}{total\ mass\ of\ element\ in\ pearling\ fraction\ or\ pearled\ grain\ (g\ dm)}\ x\ 100$$

2.3 Imaging of mineral distribution

Transverse cross sections (85 µm) of the central part of the ungerminated sample and samples A and B were obtained as in De Brier et al. (2016) and Lemmens et al. (2018). The freeze-dried radicles and coleoptiles were sandwiched between two layers of adhesive Kapton polyimide tape.

XRF maps were collected at the hard X-ray microprobe beamline P06 at the PETRA III synchrotron facility (DESY, Hamburg, Germany) as in Lemmens et al. (2018). Briefly, we used the beamline's cryogenically cooled double crystal Si (111) monochromator for X-ray energy selection and a pair of Kirkpatrick–Baez mirrors, which were focused to a spot size of 0.6 µm

(horizontal) x 0.4 µm (vertical) at a flux of 2 x 10^{10} ph/s on the sample. For XRF mapping, the incident beam energy was at 11 keV and the full XRF spectrum for each image pixel was collected using the Maia 384C detector system. The response of the detector was calibrated using standard titanium, chrome, Fe, Zn and Cu foils (MicroMatter, Surrey, Canada).

The study samples were raster scanned in on-the-fly mode with an encoded increment (pixel size) of 1 µm and a transit time per pixel of 0.3 ms for mapping the entire cross sections and the radicles and coleoptiles.

Detailed images of the distribution of mineral elements in the wheat crease regions (nucellar projection and vascular bundle) were made with increments of 0.5 µm at 2.5 ms.

Finally, detailed images were made with increments of 0.25 µm (very high resolution maps through deliberate oversampling) at 20 ms to visualise the elemental distribution within aleurone cells.

Cross sections from three independently sectioned (un)germinated wheat grains were analysed.

Semi-quantitative estimations of K, P, S, Ca, Fe, Zn, Cu, and Mn contents were made with GeoPIXE v7.4j software (Ryan et al., 1990), assuming a glucose ($C_6H_{12}O_6$) matrix with a thickness of 85 µm and density of 0.8 g/cm^3, incorporating corrections for self-absorption and absorption in air. The minimum detection limits (estimated at 99% confidence) for P, S, Fe and Zn were 50, 20, 0.2 and 0.2 ppm, respectively.

2.4 Iron and zinc X-ray absorption near-edge structure imaging

XANES spectroscopic imaging was used to investigate the chemical speciation of Fe and/or Zn in the aleurone cells, crease region and the radicles and coleoptiles in the different samples. In essence, the areas were imaged repeatedly with the photon energy gradually increasing across the absorption edge of Fe and Zn, thereby exciting core electrons in the atoms, resulting in characteristic fluorescence signals.

XANES imaging "stacks" were obtained by scanning the area of interest 104 times with increments of 3.0 μm at 1.0 ms while progressively increasing the incident X-ray energy from 7,064 to 7,269 eV across the Fe K-edge or 103 times while increasing the energy from 9,611 to 9,816 eV across the Zn K-edge. The full energy range was divided into sub-ranges, with energy step sizes in each sub-range selected between 0.5 eV across the edge to 20 eV at highest energies. From these stacks of XRF maps, XANES spectra could be extracted for each pixel. In order to improve signal quality the sum of all spectra from pixels in areas of similar sample structure were taken.

Ferrous (phytate Fe^{2+}, cysteine Fe^{2+} and citrate Fe^{2+}), ferric (phytate Fe^{3+}, cysteine Fe^{3+} and citrate Fe^{3+}), and Zn (phytate Zn^{2+}, cysteine Zn^{2+} and citrate Zn^{2+}) standards were prepared as in Lemmens et al. (2018). In essence, equal volumes of 30 mM $FeSO_4$, $FeCl_3$ and $ZnSO_4$ were mixed with 33 mM ligand in Milli-Q water adjusted to pH 2.0 with 12 M HCl. Next, the pH was adjusted to 6.0 with 0.1 M KOH to ensure complete chelation of the mineral elements. The pH of the standards containing cysteine was increased only to 4.5 to avoid oxidation to cystine. The Fe^{2+} standards were prepared in a N_2-filled anaerobic chamber using deoxygenated Milli-Q water. Finally, the standard solutions were absorbed onto narrow strips of 11 μm thick filter paper (Whatman Grade 1 filter, GE Healthcare Life Sciences, Buckinghamshire, UK), allowed to dry, sandwiched in Kapton polyimide tape to protect them from oxidation and scanned across the relevant absorption edges as outlined above.

All obtained data were processed and analysed using GeoPIXE v7.4j software and Athena v 0.9.25 software. XANES spectra of Fe and Zn reference foils were collected to calibrate the inflection point energy of the spectra.

2.5 Statistical analysis

Data were processed using the JMP Pro 14 software (SAS Institute, Cary, NC). One-way analysis of variance (ANOVA) with the Tukey multiple comparison procedure was performed

to identify significant differences ($P < 0.05$) between mean values of the white line and inflection point energy values.

3. RESULTS AND DISCUSSION

3.1 Impact of germination on the mineral content and extractability in different wheat pearling fractions

While UGP0-5% of wheat mainly consists of pericarp, UGP5-10% is clearly enriched in aleurone tissues. UGP10-15% already contains substantial portions of starchy endosperm since removal of the outer layers by abrasion was rather inhomogeneous and caused different botanical tissues to be present in the different pearling samples as was stated by De Brier et al. (2015).

UGP0-5% had the highest Zn [69 ± 1 mg/kg dry matter (dm)] and Fe (127 ± 3 mg/kg dm) contents indicating that the mineral-rich aleurone also partly ended up in this fraction (Table 1). These findings are in line with De Brier et al. (2015) and Liu et al (2008). They reported that UGP0-3% and UGP0-5% of wheat have Zn concentrations of 53 ± 1 and 47 mg/kg dm, respectively and Fe concentrations of 126 ± 2 and 119 mg/kg dm.

UGP5-10% was also relatively high in Zn (61 ± 1 mg/kg dm) and Fe (124 ± 4 mg/kg dm) and contained the highest levels of S (1,777 ± 36 mg/kg dm) and P (8,944 ± 59 mg/kg dm). De Brier et al. (2015) similarly observed relatively high concentrations of Zn (58 ± 4 mg/kg dm), Fe (103 ± 2 mg/kg dm) and P (9,100 ± 230 mg/kg dm) in wheat UGP6-9%. Further, Liu et al. (2008) found Zn and Fe concentrations of 43 and 114 mg/kg dm, respectively, in wheat UGP5-10%.

Overall, this study and earlier studies show the concentrations of mineral elements generally decreased from the outside to the inside of the wheat grain (Lu et al., 2013; Ozturk et al., 2006).

UGP0-10% and the pearled grain (*i.e.* 75% of grain dm remaining after pearling ungerminated wheat) contained 32% and 40% of all Zn, respectively. Of the sample A grain's Zn, only 23% was present in GP0-10% and only 21% in the pearled grain (*i.e.* 55% of grain dm remaining after removing radicle and coleoptile and then pearling sample A), while 27 and 13% was found

in the radicle and coleoptile (Table 1). The radicle had the highest Zn content (81 ± 8 mg/kg dm) of all fractions studied. Ozturk et al. (2006) noted Zn concentrations of up to 200 mg/kg dm after 36 h of germination in the radicle and coleoptile of wheat, indicating substantial mobilisation of Zn to the developing seedling (Takahashi et al., 2009).

The same trend was observed for Fe. Indeed, UGP0-10% and the pearled grain of ungerminated wheat contained 32 and 38% of all Fe present, while their counterparts of sample A contained only 12 and 19%, respectively. The radicle and coleoptile of sample A contained 26 and 17% of all Fe, respectively. The Fe contents in radicle and coleoptile amounted to 89 ± 1 and 58 ± 2 mg/kg dm, respectively. Li et al. (2012) reported Fe concentrations of 140 and 64 mg/kg in radicle and coleoptile of wheat germinated for 4 days at 25 °C, respectively. Fe probably directly originates from scutellum tissue attached to the seedling (De Brier et al., 2016) (*cfr*. 3.2.3).

Further, GP0-10% contained less P than UGP0-10%. This may indicate germination associated hydrolysis of phytate and translocation of P to the seedling. It is reasonable to assume that hydrolysis of phytate contributes to migration of Fe and Zn to the seedling.

Further, the S content in the starchy endosperm was 67% in ungerminated wheat but 42% in sample A, while 15 and 20% of all S was present in the radicle and coleoptile, indicating that it is transported to the developing seedling. S in (un)germinated wheat was dominantly present in the endosperm since the S-containing amino acids cysteine and methionine are part of the gluten protein structures (Eagling et al., 2014; Persson et al., 2009).

Endogenous phytase hydrolyses phytate during wheat germination (Lemmens et al., 2018). Here, we studied the implications of phytate breakdown on the water extractability and thus mobility of mineral elements within the grain tissues.

The extractability of Zn and Fe elements amounted to only 1-4% in all pearling fractions of ungerminated wheat, while it was 10-13% for S and 25-28% for P (Table 1). The Fe and Zn

elements in ungerminated wheat were clearly not extractable in water due to their chelation to phytate and their entrapment in aleurone cells with rigid walls (Lemmens et al., submitted). It is further of note that the extractability of Zn from the pearled grain (*i.e.* 75% of grain dm remaining after pearling ungerminated wheat) (4%) was slightly higher than that of the other fractions (2%), which confirms the hypothesis that part of the Zn is soluble when occurring in complex with NA and/or bound by thiol groups of cysteine-containing peptides in the starchy endosperm (Eagling et al., 2014). Interestingly, the water extractability of Zn in GP0-15% (7-8%) was 3 to 5 times higher than in UGP0-15% indicating that Zn elements became mobile during germination (Table 1).

In addition, the water extractability of S in GP0-15% was substantially higher (25-30%) than that from UGP0-15% (11-13%). One can assume that part of the Zn is complexed with soluble S-containing peptides also during translocation (Persson et al., 2016).

In contrast, the extractability of Fe increased only from 2-3% in UGP0-15% to 3-4% in GP0-15%, suggesting that Fe is less mobile than Zn during germination(Lu et al., 2013).

Takahashi et al. (2009) stated earlier that Fe and Zn translocate from rice endosperm to the scutellum and are then transported to the growing seedling during germination. In addition, Ozturk et al. (2006) found that Zn has migrated already after 36 h of wheat germination into the developing radicle and coleoptile.

Further, as a result of phytate hydrolysis during germination, the water extractability of P in GP5-10% slightly increased. Finally, it is of note that especially Zn in the radicle and coleoptile had a relatively high extractability (26-27%).

3.2 Elemental distribution in (un)germinated wheat tissues

3.2.1 Elemental distribution in the pericarp, aleurone and starchy endosperm of (un)germinated wheat grains

Comparison of the entire cross sections of ungerminated wheat and samples A and B using tri-colour high-resolution synchrotron XRF microscopy images (Figure 1) showed that germination leads to detachment of the pericarp from the aleurone as a result of the presence of *de novo* synthesised endoxylanases (Lemmens et al., submitted) acting on cell walls of the nucellar epidermis. Indeed, the arabinoxylan in the nucellar epidermis is very susceptible to enzymatic attack because of its low arabinose to xylose ratio (Van Craeyveld et al., 2010).

We here show representative results of high quality cross sections. Tri-colour images of replicates can be found in supplementary data (Figure S1).

The profiles in Figure 1 represent average elemental concentrations (mg/kg) in the areas indicated by the boxes in the tri-colour images. The corresponding µ-XRF elemental maps can be found in supplementary data (Figure S2). Although Ca was mainly present in the aleurone (AL, 100 to 200 µm section), it was also clearly present in the pericarp (P, 25 to 100 µm section) and starchy endosperm (SE, 200 to 350 µm section) of ungerminated wheat (De Brier, Gomand, et al., 2015) (Figure 1). As noted above, Ca has low mobility in the phloem and may also enter the grain through direct xylem supply of the pericarp. It is equally possible that it reaches the pericarp through the phloem as a result of xylem discontinuity in the grain stalk (De Brier et al., 2016; Pearson et al., 1999; White, 2012). Fe and Zn, which both have at least moderate phloem mobility (see above), were mainly present in the region corresponding to the aleurone.

The co-localisation of Fe, Zn and Ca with P and S was studied to gain more insight in their binding with ligands such as phytate and peptides/proteins. P was the most abundant mineral element under study and mainly present in the aleurone region (Figure 1). Fe and Zn clearly co-

301 localised with P suggesting their chelation by phytic acid in wheat (Lemmens et al., 2018). The
302 aleurone also contained relatively high concentrations of S, which can be present as S-
303 containing peptides/proteins. Ca (1.0-1.5 g/kg) and other trace elements (Fe and Zn about 20
304 and about 10 mg/kg, respectively) in the starchy endosperm were probably not bound to phytate.
305 Much as Persson et al. (2016) we found only very low concentrations of P in this tissue. In the
306 starchy endosperm, Fe and Zn occur mostly as soluble complexes with NA, deoxymugineic acid
307 and S-containing peptides (Eagling et al., 2014; Persson et al., 2016; Xue et al., 2014). Figure 1
308 confirms that S was also dominantly present in the starchy endosperm. Here it is mainly part of
309 the amino acids cysteine and methionine (de J. Mangueze et al., 2018).

310 After steeping and germination, Fe and Zn were still mainly localised in the aleurone cells (AL,
311 90 to 160 µm in sample A and AL, 140 to 200 µm in sample B of Figure 1). Although Fe was
312 not translocated, Zn partly (15-30%) migrated to the pericarp both when germination had been
313 at 15 °C (sample A, P 20 to 90 µm section) or 26 °C (sample B, P 30 to 110 µm section). As
314 noted above, Zn is an important mineral element during seedling development and one can
315 assume that it is partly passively transported to the embryo through the pericarp. Although Zn
316 partly migrated from the aleurone to the pericarp once released from its phytate structures, there
317 was no significant difference ($P < 0.05$) in Zn concentrations between whole meal from
318 ungerminated (24 ± 1 mg/kg dm) and from germinated wheat (26 ± 3 mg/kg dm, sample A) and
319 almost no leaching of Zn into the steeping medium (data not shown). It is equally possible that
320 the released Zn elements, when in excess, are diverted from the endosperm and embryo to the
321 pericarp to avoid toxicity stress (Pearson et al., 1999).

322 In contrast, no changes in Ca, P and S distributions were observed as a result of germination.
323 Although 15-33% of phytate was broken down (Lemmens et al., submitted), P was not
324 translocated through the wheat grain during germination. However, in section 3.1 we observed
325 that 28% of all P in sample A was present in the seedling which, in line with observations for

Fe, probably directly originated from scutellum tissue attached to the seedling (De Brier et al., 2016).

The elemental distribution within a few aleurone cells (about 40 x 40 µm each) of an ungerminated wheat grain (Figure 2), sample A (Figure S3, supplementary data) and sample B (Figure S4, supplementary data) were also visualised at very high resolution (0.25 µm) (*cfr.* 2.4).

From Figure 2, it is clear that P was confined in individual spherical globoids together with Fe and Zn while little, if any, Fe and Zn elements were detected in the aleurone cell walls. S was mainly present in the endosperm (left part in S elemental map of Figure 2) where, as stated above, it is present in cysteine and methionine amino acid residues.

3.2.2 Elemental distribution in the crease region of (un)germinated wheat grains

We here studied the distribution of Fe, Zn, P and S in the crease region of (un)germinated wheat grains (Figure 3).

The nucellar projection in the ungerminated wheat grain contained high Fe and S levels, while there was almost no Zn and P, which is in line with the findings of De Brier et al. (2016) and Singh et al. (2014). The present data suggest that Fe is not chelated to phytate during transport and that it is bound to S-rich protein in the nucellar projection (De Brier et al., 2016; Singh et al., 2014). In contrast, Zn was clearly present in the nucellar projection of sample B. We hypothesise that Zn, not in complex with phytic acid, may also partly translocate to the developing embryo through the cells of the nucellar projection running through the crease of the grain.

The Fe and especially the P levels in the vascular bundle (transfer cells) of both ungerminated and germinated wheat grains (samples A and B) were rather low. In contrast, its Zn and S levels were relatively high, especially in the germinated wheat grains. For barley it has been reported

that Zn is released from its phytate structures during germination, becomes mobile and is transported bound to S-containing peptides through, amongst others, these transfer cells, as many genes coding for Zn transporters are present in the vascular bundle and as it contains vacuoles in which Zn is stored (Tauris et al., 2009).

The present findings lend support to the statement by Singh et al. (2014) that the crease region in wheat plays a crucial role in element translocation and accumulation.

The aleurone cells surrounding the crease (*i.e.* the modified aleurone cells) were in shape and size similar to the regular aleurone cells and both before and after germination contained high levels of Fe, Zn and P.

S was mainly present in the peripheral starchy endosperm cells.

3.2.3 Elemental distribution in the seedling of germinated wheat grains

To the best of our knowledge, the present study is the first which with μXRF microscopy visualises the distribution of mineral elements in a developing wheat radicle and coleoptile. The insights generated can contribute to a better understanding of the physiological role of mineral elements during plant development.

K was present in the radicle and coleoptile in high concentrations (Figure 4). Earlier, Lu et al. (2013) observed that K is dominantly present in the radicle and embryo of non-germinated rice. K has an important role in the activation of many growth related enzymes and may be important for allowing growing cells to maintain turgor pressure (Lu et al., 2013).

In line with the report by Ozturk et al. (2006), we found Zn to be clearly concentrated in the radicle tip. Ozturk et al. (2006) also observed its accumulation in the coleoptile and radicle tip where it is crucially needed for protein synthesis, structural integrity and stress tolerance (Marschner, 2012).

Ca levels were increased in the elongating coleoptile tip. Lu et al. (2013) also observed clear mobilisation of Ca to the rice coleoptile where it is involved in cell wall structure and cell growth and plant cell signalling.

Finally, the remnants of the scutellum attached to the coleoptile contained high concentrations of K, Mn, Zn and Fe (Sample A in Figure 4 and Figure S5, supplementary data). The scutellum has a role in exchange of nutrients between embryo and endosperm (Marschner, 2012). Earlier De Brier et al. (2016) found the scutellum to be rich in Fe and Mn.

The germination temperature (15 °C versus 26 °C) did not influence the distribution of mineral elements in radicle or coleoptile.

3.3 Impact of germination on the iron speciation in different wheat tissues

The energies of the inflection point of the absorption edge (*i.e.* maximum of the spectrum's first derivative) and the white line (*i.e.* principal peak) of the ferric phytate standard in the XANES spectra were at 7,128.5 eV and 7,131.8 eV, while these values for the ferrous phytate standard were at 7,124.5 eV and 7,131.2 eV (Figure 5.1A). The inflection point is 1 to 4 eV higher for the ferric than for the ferrous form (De Brier et al., 2016; Lemmens et al., 2018).

During germination, no Fe oxidation occurred in the aleurone cells since their inflection point and white line were at 7,124.5 eV and 7,130.1 in ungerminated wheat, while they were at 7,124.5 eV and 7,130.3 eV for aleurone cells of both samples A and B, respectively (Figure 5.1B). This is unlike what was earlier observed by Lemmens et al. (2018). They found the inflection point of Fe in the germinated wheat grain (sample A) at a significantly higher ($P < 0.05$) energy (7128.1 eV), and thereby thus provided evidence of Fe oxidation during germination of wheat.

The inflection point and white line positions of modified aleurone cells were at 7,124.0 eV and 7,131.0 eV and of the nucellar projection, at 7,127.0 eV and 7,132.0 eV, respectively (Figure

5.1B). These results suggest that modified aleurone cells contained mainly ferrous species and that the nucellar projection, which had a higher inflection point energy, contained mainly ferric species. Earlier, De Brier et al. (2016) suggested that both ferrous and ferric species co-occur in the modified aleurone cells and that the nucellar projection contains mainly the ferric Fe form. Germination did not significantly affect these energy positions in the crease region ($P < 0.05$).

Comparison of the XANES spectra of the standards (Figure 5.1A) with those of the study samples (Figure 5.1B), clearly showed that phytate standards exhibited a typical shoulder feature at 7,138.0 eV which was also present in the spectra extracted from aleurone and modified aleurone cells of the (un)germinated wheat samples. Although phytate was partly hydrolysed during germination [15-33% losses (Lemmens et al., submitted)], Fe still mainly occurred as phytate chelates. Neal et al. (2013) fitted phytate models for wheat using extended X-ray absorption fine structure spectroscopy and stated that Fe in (modified) aleurone cells conformed to these models. As the Fe XANES spectra of the nucellar projection in (un)germinated wheat did not contain the typical phytate features, the chemical environment of Fe differed from those in the (modified) aleurone cells. It was previously shown that Fe in the nucellar projection is mainly bound to citrate, NA and FeO(OH) (De Brier et al., 2016).

3.4 Impact of germination on the zinc speciation in different wheat tissues

The spectral white line and overall shape of the spectra of Zn standards containing phytate, cysteine, sulfate and citrate were here compared. The Zn-phytate standard had a white line position at 9,662.3 eV and exhibited a shoulder feature at 9,667 eV (Figure 5.2A). The Zn-cysteine standard showed a wide white line peak from 9,662 to 9,667 eV and had a centre white line position at 9,665.2 eV. The shapes of the spectra of the Zn-sulfate and Zn-citrate standards were similar, while the white line position of Zn-sulfate was at 9,665.7 eV and that of Zn-citrate at 9,665.0 eV.

The energy positions of the white line of the Zn XANES spectra of the (modified) aleurone cells of ungerminated wheat, sample A and sample B were all at 9,662.0 eV. In addition, all these spectra exhibited a clear shoulder feature at energies between 9,666 and 9,668 eV (Figure 5.2B). However, the peak of this shoulder feature in the study samples of the (modified) aleurone cells was more pronounced than that in the Zn-phytate standard. This suggests that part of Zn is bound to sulfate compounds (for example to sulfate micronutrient transporters) (Buchner et al., 2010) and/or cysteine which both have peaks at energies corresponding to the shoulder feature of the spectra derived from (modified) aleurone cells. It is noted here that the shoulder feature is less pronounced for (modified) aleurone cells of sample B than that of ungerminated wheat and sample A, indicating that germination impacts Zn speciation and that other Zn species occur in these cells.

The energy positions of the white line of the Zn XANES spectra of the vascular bundle of ungerminated wheat, sample A and sample B were all at 9,664.0 eV. These spectra no longer showed the shoulder feature, indicating that the chemical environment of Zn was different from that in the (modified) aleurone cells. The white line and shape of the spectra of the vascular bundle in (un)germinated wheat were comparable to those of the Zn-cysteine standard. Persson et al. (2016) found that Zn can be bound to soluble S-containing peptides. As a result, it seems logical that it is very mobile, which facilitates its migration to the embryo through the transfer cells in the vascular bundle (or passively through the pericarp).

The XANES spectra of the radicle and coleoptile samples (Figure 5.2C) were different from those of the (modified) aleurone cells and vascular bundle. We here hypothesise that Zn was neither phytate nor cysteine bound and that other ligands such as NA and methionine may have been in play. It is also possible that after transportation of Zn to the metabolically active seedling, it is bound to cell wall specific compounds (for example phenolic compounds) (Marschner, 2012).

4 CONCLUSIONS

This study unravelled the impact of wheat germination on mineral translocation and chemical speciation of Fe and Zn.

During germination, Zn is clearly translocated from aleurone tissues to the developing embryo, especially the radicle where it has a high water extractability (27%) and thus high mobility. It is hypothesised that it is passively transported through the pericarp and/or selectively transported through the transfer cells in the vascular bundle and nucellar projection since relatively high concentrations of Zn are detected in these tissues as a result of germination. Co-localisation and speciation analysis showed that Zn elements are probably transported as and accumulate in complex with S-containing compounds such as sulfate and/or protein. In contrast, Fe is translocated from aleurone tissues to the developing embryo, where it has a low water extractability (4-5%) and thus low mobility. It is predominantly present in scutellum remnants attached to the seedling. Although 15-33% of phytate is broken down during germination, at least a significant portion of Fe elements remain present in the (modified) aleurone as phytate chelates. P is only to a limited degree translocated throughout the grain and remains mainly in the aleurone.

To conclude, the changes in water extractability of mineral elements after germination are rather related to migration to another tissue than to changes in their chemical speciation within the tissue. The gathered results can be used for further identifying chelating compounds involved in transport and deposition of mineral elements during germination of wheat grains. Moreover, they can be used to substantiate changes in mineral bio-accessibility upon wheat germination.

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568

569 Table 1 The role of germination on water extractability of Zn, Fe, S and P. Total concentrations of these elements and their water extractabilities are
570 given for wheat pearlings (P) of different extent (% mass pearled given) and in pearled grains of ungerminated wheat and of wheat steeped (15 °C, 29
571 h) and germinated (15 °C, 120 h) (sample A). Data are means ± standard deviation of two independent replicates each measured with at least 4
572 technical repeats.

	Mass fraction (%)		**Zn content (mg/kg dm)**		**Zn extractability (%)**		**Fe content (mg/kg dm)**		**Fe extractability (%)**	
	Ungerminated wheat	**Sample A**	**Ungerminated wheat**	**Sample A**	**Ungerminated wheat**	**Sample A**	**Ungerminated wheat**	**Sample A**	**Ungerminated wheat**	**Sample A**
P0-5%	5.0	5.0	68.8 ± 0.7	90.4 ± 1.0	1.8 ± 0.1	7.8 ± 1.4	127 ± 3.0	40.6 ± 0.9	2.0 ± 0.1	3.6 ± 0.3
P5-10%	5.0	5.0	61.3 ± 0.8	49.1 ± 2.6	1.5 ± 0.1	7.9 ± 0.6	124 ± 4.0	53.4 ± 1.8	2.7 ± 0.1	3.1 ± 0.2
P10-15%	5.0	5.0	44.7 ± 0.9	40.1 ± 1.7	2.3 ± 0.1	6.8 ± 0.1	87.7 ± 1.2	59.7 ± 8.6	2.2 ± 0.1	3.0 ± 0.1
P15-25%	10.0	10.0	32.8 ± 0.9	31.6 ± 1.7	3.0 ± 0.1	6.8 ± 0.2	74.3 ± 2.6	49.5 ± 7.0	1.2 ± 0.2	1.4 ± 0.1
Pearled grain	75	55	10.8 ± 0.7	11.6 ± 0.3	3.9 ± 0.1	8.0 ± 0.1	19.6 ± 1.3	11.8 ± 1.0	1.0 ± 0.1	1.3 ± 0.2
Radicle		10.0		81.0 ± 8.0		25.9 ± 1.4		89.2 ± 0.1		4.1 ± 0.1
Coleoptile		10.0		38.1 ± 0.3		27.2 ± 1.8		58.2 ± 2.1		4.9 ± 0.1
Total content (mg/ kg dm)			23.6 ± 1.3	26.2 ± 2.6			34.8 ± 0.9	34.0 ± 1.5		
Mass balance (%)	100	100	86	116			112	100		

573 Table 1 Continued.

	Mass fraction (%)		S content (mg/kg dm)		S extractability (%)		P content (mg/kg dm)		P extractability (%)	
	Ungerminated wheat	**Sample A**	**Ungerminated wheat**	**Sample A**	**Ungerminated wheat**	**Sample A**	**Ungerminated wheat**	**Sample A**	**Ungerminated wheat**	**Sample A**
P0-5%	5.0	5.0	1,611 ± 9	1,532 ± 45	12.6 ± 0.2	24.9 ± 0.9	8,210 ± 99	7,773 ± 124	25.4 ± 0.6	23.7 ± 0.7
P5-10%	5.0	5.0	1,777 ± 36	848 ± 26	11.3 ± 0.3	30.0 ± 0.3	8,944 ± 59	4,544 ± 195	26.5 ± 0.3	28.6 ± 0.1
P10-15%	5.0	5.0	1,586 ± 34	878 ± 20	11.1 ± 0.1	28.9 ± 0.5	7,408 ± 220	4,998 ± 79	26.5 ± 0.1	29.7 ± 0.8
P15-25%	10.0	10.0	1,480 ± 43	1,179 ± 33	10.5 ± 0.1	18.5 ± 0.1	5,585 ± 46	4,639 ± 43	28.4 ± 0.6	25.6 ± 1.3
Pearled grain	75	55	1,072 ± 9	920 ± 1	9.8 ± 0.1	17.0 ± 0.2	1,938 ± 96	2,311 ± 26	18.8 ± 1.5	27.2 ± 0.1
Radicle		10.0		1,860 ± 18		18.8 ± 0.2		5,564 ± 154		44.8 ± 0.7
Coleoptile		10.0		2,456 ± 16		16.2 ± 0.2		4,533 ± 100		43.1 ± 1.0
Total content (mg/ kg dm)			1,378 ± 32	1,256 ± 22			3,463 ± 132	3,487 ± 232		
Mass balance (%)	100	100	87	97			94	104		

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FIGURE CAPTIONS

Figure 1 Tri-colour images of zinc (Zn; red), phosphorus (P; green) and calcium (Ca; blue) distribution in cross sections of grains of ungerminated wheat, wheat germinated for 120 h at 15 °C (sample A) and wheat germinated for 120 h at 26 °C (sample B). Elemental concentration profiles of P, S, Ca, Fe and Zn from a selected area (white box in figure) are shown from the outside to the inside and thus pericarp (P), aleurone (AL) and starchy endosperm (SE). The data were obtained by using the transect function in GeoPIXE over a distance of 350 µm wide starting at the outer side.

Figure 2 µ-XRF elemental maps of phosphorus (P), iron (Fe), zinc (Zn) and sulfur (S) in aleurone cells of an ungerminated wheat grain. The colour scale represents relative concentrations.

Figure 3 Tri-colour images of (A) iron (Fe, red), sulfur (S, green) and phosphorus (P, blue) and (B) zinc (Zn, red), sulfur (S, green) and phosphorus (P, blue) distribution in the crease region [modified aleurone cells (MA), nucellar projection (NP) and vascular bundle (VB)] in cross sections of grains of ungerminated wheat, wheat germinated for 120 h at 15 °C (sample A) and wheat germinated for 120 h at 26 °C (sample B).

Figure 4 Tri-colour images of zinc (Zn, red), potassium (K, green) and calcium (Ca, blue) distribution in radicle and coleoptile of wheat grains germinated for 120 h at 15 °C (sample A) or 120 h at 26 °C (sample B).

Figure 5.1 iron X-ray absorption near-edge structure (XANES) spectroscopy spectra of (A) standard references and (B) spectra of aleurone cells (AL), modified aleurone cells (MA) and nucellar projection (NP) selected in cross sections of ungerminated wheat, wheat germinated for 120 h at 15 °C (sample A) and wheat germinated for 120 h at 26 °C (sample B).

Figure 5.2 zinc X-ray absorption near-edge structure (XANES) spectroscopy spectra of (A) standard references, (B) spectra of aleurone cells (AL), modified aleurone cells (MA) and

vascular bundle (VB) selected in cross sections of ungerminated wheat, wheat germinated for 120 h at 15 °C (sample A) and wheat germinated for 120 h at 26 °C (sample B) and (C) spectra of radicle cells (R) and coleoptile cells (C) selected in wheat germinated for 120 h at 15 °C (sample A) and wheat germinated for 120 h at 26 °C (sample B).

FIGURE CAPTIONS SUPPLEMENTARY DATA

Figure S1 Tri-colour images of zinc (Zn; red), phosphorus (P; green) and calcium (Ca; blue) distribution in cross sections of grains of ungerminated wheat, wheat germinated for 120 h at 15 °C (sample A) and wheat germinated for 120 h at 26 °C (sample B).

Figure S2 µ-XRF elemental maps of cross sections of ungerminated wheat, wheat germinated for 120 h at 15 °C (sample B) and wheat germinated for 120 h at 26 °C (sample B). The colour scale represents relative concentrations.

Figure S3 µ-XRF elemental maps of phosphorus (P), iron (Fe), zinc (Zn) and sulfur (S) in aleurone cells of a wheat grain germinated for 120 h at 15 °C (sample A). The colour scale represents relative concentrations.

Figure S4 µ-XRF elemental maps of phosphorus (P), iron (Fe), zinc (Zn) and sulfur (S) in aleurone cells of a wheat grain germinated for 120 h at 26 °C (sample B). The colour scale represents relative concentrations.

Figure S5 Tri-colour image of manganese (Mn, red), iron (Fe, green) and copper (Cu, blue) distribution in the scutellum attached to coleoptile of a germinated wheat grain (sample A). We want to note that the Mn, Fe and Cu concentrations in the remainder of the coleoptile are low.