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Concentration and localization of zinc during seed development and germination in wheat

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In a field experiment, the effect of foliar Zn applications on the concentration of Zn in seeds of a bread wheat cultivar (*Triticum aestivum* L. cv. Balatilla) was studied during different stages of seed development. In addition, a staining method using dithizone (DTZ: diphenyl thiocarbazone) was applied to (1) study the localization of Zn in seeds, (2) follow the remobilization of Zn during germination, and (3) develop a rapid visual Zn screening method for seed and flour samples. In all seed development stages, foliar Zn treatments were effective in increasing seed Zn concentration. The highest Zn concentration in the seeds was found in the first stage of seed development (around the early milk stage); after this, seed Zn concentration gradually decreased until maturity. When reacting with Zn, DTZ forms a red-colored complex. The DTZ staining of seed samples revealed that Zn is predominantly located in the embryo and aleurone parts of the seeds. After 36 h of germination, the coleoptile and roots that emerged from seeds showed very intensive red color formation and had Zn concentrations up to 200 mg kg^{-1}, indicating a substantial remobilization of Zn from seed pools into the developing roots (radicle) and coleoptile. The DTZ staining method seems to be useful in ranking flour samples for their Zn concentrations. There was a close relationship between the seed Zn concentrations and spectral absorbance of the methanol extracts of the flour samples stained with DTZ. The results suggest that (1) accumulation of Zn in seeds is particularly high during early seed development, (2) Zn is concentrated in the embryo and aleurone parts, and (3) the DTZ staining method can be used as a rapid, semiquantitative method to estimate Zn concentrations of flour and seed samples and to screen genotypes for their Zn concentrations in seeds.

Introduction

Increasing the concentration of micronutrients (especially Zn) in food crop plants is a growing global challenge, with potentially great implications for both crop production and human health. It is believed that

Abbreviations – DTPA, diethylenetriaminepentaacetate; DTZ, diphenyl thiocarbazone; ICP-OES, inductively coupled argon plasma optical emission spectrometer.

Zn deficiency is the most widespread micronutrient deficiency in crop plants and human beings (Alloway, 2004; Hotz and Brown, 2004). Zn deficiency in humans causes a wide range of health complications, including impairments in the immune system, learning ability and physical growth, and increases in mortality and infections (Hotz and Brown, 2004; Cunnigham-Rundles et al., 2005). Zinc deficiency also induces DNA damage and increases the risk of cancer occurrence (Ho, 2004).

About a half of the cereal-growing areas in the world contain low levels of plant-available Zn in the soil, and the plants grown in such areas suffer from Zn deficiency stress and contain low levels of Zn in the grain (Graham and Welch, 1996; Cakmak et al., 1999). Cereals (e.g. wheat, rice and maize) with inherently low Zn concentrations in the grain are the most important source of calories in the developing world. High consumption of cereal-based foods over long periods may induce Zn deficiency problems, resulting in severe health complications. To meet the daily Zn requirement of humans, the amounts of Zn in the seed need to exceed the optimum level for the crop itself (Welch, 1999; Rengel et al., 1999; Grusak and Cakmak, 2005). Milling of cereal grains is another concern with regard to low levels of Zn in cereal-based food products. During the milling process, a significant proportion of Zn within the seed is lost, due to the removal of Zn-rich bran, which consists of the aleurone and embryo (Welch, 1986; Welch and Graham, 1999).

Currently, improving the grain Zn concentration of cereal crops is a high-priority research area. Among the strategies discussed in the literature, agricultural approaches (e.g. breeding, application of fertilizers) seem to be the most promising and sustainable solution to the Zn deficiency problem (Cakmak et al., 2002; Welch and Graham, 2004; White and Broadley, 2005). As shown in Central Anatolia, the combination of soil and foliar application of Zn fertilizers to cereal crops appears to be a practical and effective way of maximizing root uptake and grain accumulation of Zn (Yilmaz et al., 1998). There is, however, little research on the role of the amount, frequency and timing of the foliar Zn applications in increasing grain Zn concentrations (Rengel et al., 1999). Information about the dynamics of Zn accumulation in seeds could be helpful in determination of the best time for foliar Zn applications.

The present study was aimed at elucidating a role for foliar Zn application in the Zn enrichment of seeds of a bread wheat cultivar grown under field conditions. The concentration and localization of Zn within the seeds during different seed development stages were studied by a dithizone (DTZ: diphenyl thiocarbazone) staining method. The DTZ staining method was also applied to rank wheat genotypes and flour samples according to Zn concentration. DTZ is a Zn-chelating agent (McNary, 1954; Lopez-Garcia et al., 2002) and is used in the localization of Zn in different organisms, such as algae (Pawlik-Skowronska, 2003), yeast (Bilinski and Miller, 1983), salmon (Paulsen et al., 2001), and maize and wheat seeds (Haug, 1984; Ehret, 1985). In the studies carried out by Haug (1984) and Ehret (1985), Zn was shown to be largely localized in the embryo, aleurone and scutellum by the application of a DTZ staining method to one maize and one wheat cultivar. In the present study, we used a range of wheat genotypes with different concentrations of Zn and other micronutrients to determine whether the DTZ method could be useful as a rapid screening method for seed Zn concentration. This method was also applied to germinating seeds to follow Zn transport from seed into the emerging roots and coleoptile.

Materials and methods

Seeds of *Triticum aestivum* L. cv. Balatilla were grown in a field [diethylenetriaminepentaacetate (DTPA)-Zn: 0.25 mg kg^{-1} soil] located in the research farm at Cukurova University Agricultural Faculty in Adana, Turkey during the 2003–2004 cropping season. At sowing, a compound fertilizer containing 20% N, 20% P_2O_5 and 1% Zn was applied at a rate of 170 kg ha^{-1}. Nitrogen was top-dressed at early tilling (urea: 150 kg ha^{-1}) and stem elongation (ammonium nitrate: 200 kg ha^{-1}). Foliar Zn applications were done by spraying Zn, 0.68 kg ha^{-1}, on plants in the form of $ZnSO_4.7H_2O$ between the stem elongation and booting stages three or 10 times at approximately 10- or 3-day intervals.

In order to study the accumulation of Zn during different stages of seed development, seed sampling (every 3–7 days) was started 12 days after anthesis and lasted for 40 days until full ripening. Sampled seeds were dried at 45°C for 72 h, acid digested with a closed microwave system (Milestone 1200 Mega) and analyzed for Zn by using an inductively coupled argon plasma optical emission spectrometer (ICP-OES) (Jobin-Yvon, JY138-Ultrace). The same acid digestion method was applied for measurement of Zn in different parts of germinating seeds. Seeds were germinated on filter paper moistened with water in Petri dishes under room conditions. Seeds were stained after 36 h, and seed parts (roots, coleoptile, embryo and remainder of seeds) were harvested for Zn analysis after 48 h of germination. For adequate sampling of different seed parts for Zn analysis by ICP-OES, a 48-h germination period was necessary.

To study the localization and assess the concentration of Zn in the whole and germinating seeds and flour samples, a staining method was developed using DTZ, which produces a red–purple Zn–dithizonate complex (McNary, 1954). Staining of dry seed samples from different developmental stages was conducted by fixing seeds in epoxy resin. For this purpose, the bases of cylindrical teflon moulds were filled initially with a 2–3-mm-high column of epoxy resin; this was followed by incubation for partial hardening. Seeds were horizontally arranged in a row on the semi-hardened resin, and moulds were then filled with freshly prepared resin until the seeds were fully covered. When fully hardened, the resin with embedded seeds was ground with 120-, 600- and 1200-grit sandpapers successively, in order to make highly polished longitudinal sections of seeds. A water jet was directed at samples during the grinding process to prevent heating and clogging of sandpaper. On completion of grinding, polished surfaces were quickly rinsed in water and stained for 30 min in a DTZ solution that was freshly prepared by dissolving 1,5-diphenyl thiocarbazone (Merck), 500 mg L^{-1}, in analysis-grade pure methanol. Finally, samples were rinsed thoroughly in water and gently dried with tissue paper.

For staining mature seed samples, dry seeds were placed for 1 h in water prior to excision, whereas germinating seeds were directly excised longitudinally along the crease with a scalpel and stained as described above. Samples were then quickly mounted on a microscope slide with the use of adhesive paste (with the excised surfaces facing upwards) and qualitatively analyzed using a reflectance light microscope (Nicon SMZ1500) with a high-resolution digital camera (Diagnostic Instruments Inc.). To prevent drying of the sensitive roots and coleoptile, samples were kept wet during microscopy by spraying them with water every 2–3 min, and the microscopy was completed within 15 min. Extremely wet surfaces were gently dried prior to or during microscopy.

Whole seed flour samples were obtained by grinding the seeds in a vibrating agate cup mill (Fritisch-Pulverisette 9) for 5 min. Flour samples of approximately 200 mg were placed in 24-well plates, and the sample surfaces were flattened with a plastic bar prior to staining with 200 µl of DTZ solution. Samples were then incubated for 30 min for maximum color formation. The red color produced by DTZ staining was stable for at least 1 h. Fading in color was evident only after more than 3 h following the staining process.

In order to study the relationship between seed Zn concentration and DTZ staining, an additional experiment was conducted using two durum (*Triticum durum* cv. Kunduru and Selcuklu) and two bread wheat (*Triticum aestivum* cv. Bagci and Balatilla) cultivars treated with 0, 3, 6 or 9 foliar Zn applications by spraying Zn, 0.68 kg ha^{-1}, on plants in the form of $ZnSO_4.7H_2O$, as described above. The use of different cultivars and different leaf treatments led to a wide range of seed Zn concentrations (i.e. 10–55 mg kg^{-1}). Following DTZ staining of whole seed flour samples (see above), the color produced was extracted with 2 mL of methanol. All extracts were then centrifuged at 5000 *g* and light absorbance at 512 nm was measured in 1-mL aliquots using a spectrophotometer (Cary-300, Varian Inc., Australia).

Results

Foliar application of $ZnSO_4$ at different stages of seed development resulted in distinct increases in grain Zn

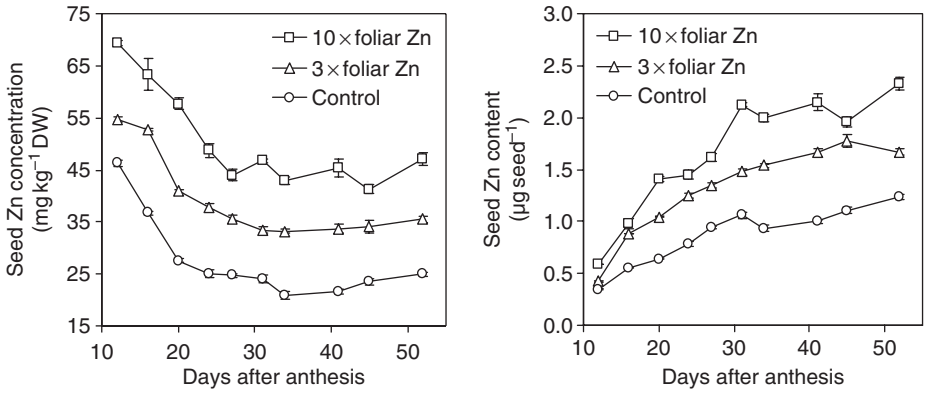

Fig. 1. Effect of three and 10 foliar Zn applications (0.68 kg Zn ha^{-1} as $ZnSO_4$) on seed Zn concentration and total amount of Zn per seed in wheat (*Triticum aestivum*, cv. Balatilla). Foliar Zn applications were initiated at early stem elongation and completed before head emergence (booting stage). Plants were grown in a Zn-adequate field. In order to follow Zn accumulation in seeds during seed development, seed sampling was initiated 12 days after anthesis and continued until seed maturation at 3–7-day intervals. Vertical bars represent ± SD of four independent replications.

concentration (Fig. 1). These increases became more pronounced with frequent foliar Zn application (10 times, every 3 days). The highest Zn concentration following foliar Zn applications was found at the beginning of seed development (around the early milk stage). Thereafter, with all treatments, seed Zn concentration declined with a similar pattern until the end of the late milk stage (27 days after anthesis), remaining generally stable until maturity (Fig. 1). During the first half of seed development (up to 27 days after anthesis: end of late milk stage), seed Zn concentration decreased by 35% in the treatment with 10 foliar Zn applications and by 45% in the control plants, mainly due to a dilution (i.e. increased biomass of seed) effect. Thereafter, the Zn concentrations of seeds did not significantly change during the rest of seed development.

As expected, the total amount of Zn per seed (Zn content) increased progressively until seed maturation, as a consequence of the increased seed size and weight (Fig. 1). In contrast to Zn concentration, the seed Zn content markedly increased, especially during the first half of seed development, and continued to increase until the end of the ripening stage.

The seeds from the field experiment with and without foliar Zn applications were used to detect the localization of Zn in seeds by staining of Zn with DTZ. When reacting with Zn, DTZ forms a red Zn–DTZ complex (Fig. 2). The formation of the Zn–DTZ complex on excised seed surfaces was more intense with higher Zn concentration in the wheat seeds. In accordance with the results shown in Fig. 1, the most intensive red color formation in seeds was seen at the beginning of seed development (Fig. 2). Differential localization of Zn in the seeds can be easily seen on the longitudinal sections of seeds. The DTZ staining method revealed that Zn is predominantly localized in the embryo and aleurone parts of the seeds (Fig. 2). With increasing seed Zn concentration, the red color produced by the Zn–DTZ complex was more intense in the embryo and aleurone parts (Fig. 3).

The red color formation with DTZ appears to be specific for Zn in wheat seeds. In order to study the effect of other metals (Cu, Mn, Cd and Fe) on the red color formation by DTZ, seeds were incubated in solutions containing increasing amounts of these metals (Fig. 4). With the exception of Cd, no red color formation was observed with the other metals up to 134 mg Cu kg^{-1}, 142 mg Fe kg^{-1} and 180 mg Mn kg^{-1} (Fig. 4). In the case of Cd, a red color developed in flour samples containing more than 15 mg Cd kg^{-1}. To our knowledge, such high Cd concentrations in cultivated crop grains have not been reported. Also, the absence of red color formation in seeds containing less than 15 mg Zn kg^{-1} indicates that other cations, such as Ca^{2+}, Mg^{2+} and K^{+}, do not interfere with the development of the red Zn–DTZ complex.

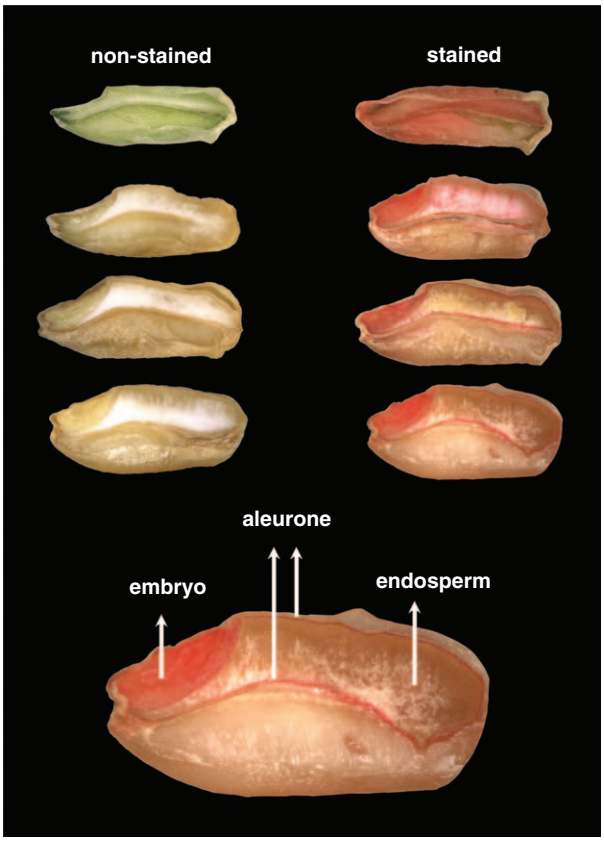

Fig. 2. Wheat (*Triticum aestivum*, cv. Balatilla) seeds at different developmental stages with and without DTZ staining. Seeds from top to bottom were sampled 12, 24, 34 and 52 days after anthesis and stained with DTZ. The seed at the bottom with labeled seed parts is the same seed sampled 52 days after anthesis (maturation stage).

Localization of Zn in the newly developing roots (radicle) and coleoptile during seed germination was visualized by using the DTZ staining method in *Triticum dicoccum* seeds containing 17 or 61 mg Zn kg^{-1}. With the development of the radicle (root) and coleoptile during seed germination over 36 h, an intensive remobilization of Zn from seed into the roots and coleoptile took place, especially in seeds with high Zn concentrations (Fig. 5). Zn was particularly concentrated in the coleoptile and root tip. Interestingly, staining of the newly developed roots was intense if they were cut longitudinally. Red color formation was less than expected in non-excised roots, although the roots contained up to 200 mg Zn kg^{-1} (Fig. 6). The reason for this observation is not understood at present.

In a separate experiment, seeds with variable total Zn concentrations were germinated for 48 h and analyzed

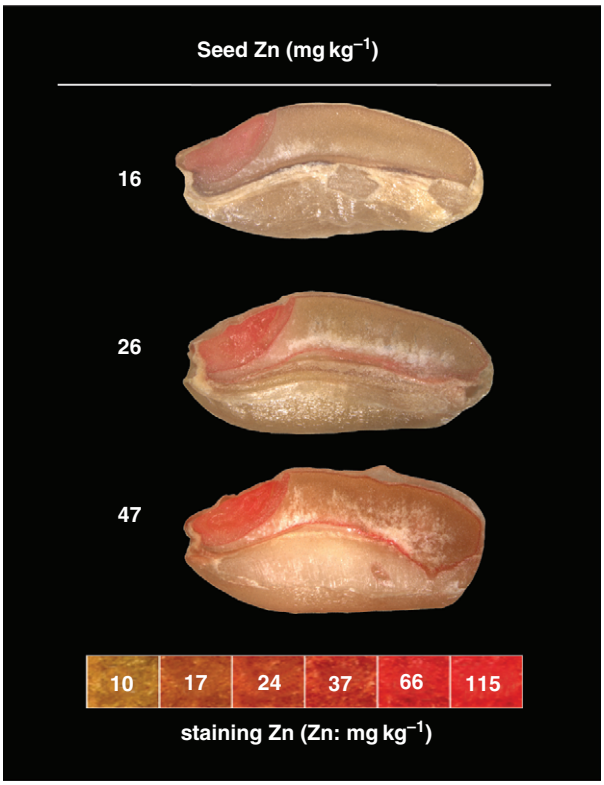

Fig. 3. DTZ staining of seeds with different Zn concentrations. Wheat (*Triticum aestivum*) cultivars used were (from top to bottom): Karacadag, Balatilla and Balatilla enriched with Zn by foliar application.

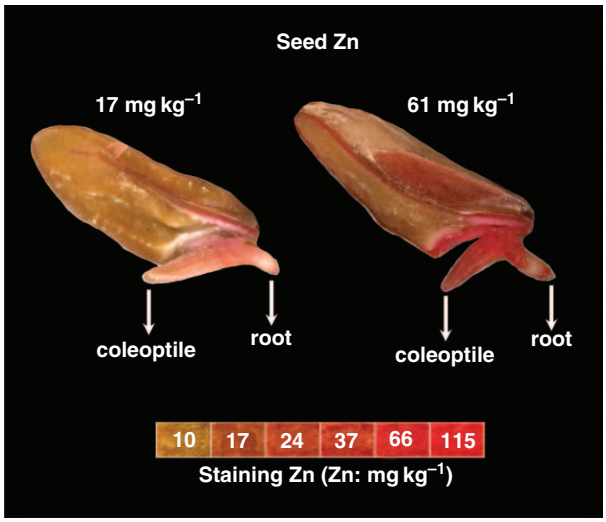

Fig. 5. DTZ staining of the germinated seeds of *Triticum dicoccum* (accession Hakan-2004) containing 17 or 61 mg Zn kg^{-1}. To obtain seeds with different concentrations of Zn, *Triticum dicoccum* seeds were grown in pots under greenhouse conditions with and without leaf application of $ZnSO_4$. Foliar Zn application [0.3% (w/v)] was done on days 30, 40, 45 and 50 after sowing. When fully mature, seeds were harvested and analyzed for Zn. Seeds were treated for DTZ staining following 36 h of germination in a Petri dish.

for Zn in the roots (radicle), coleoptile, embryo and the remainder of seeds (Fig. 6). With increase in Zn concentration from 12 to 55 mg kg^{-1}, the roots and coleoptile of the germinated seeds showed marked increases in

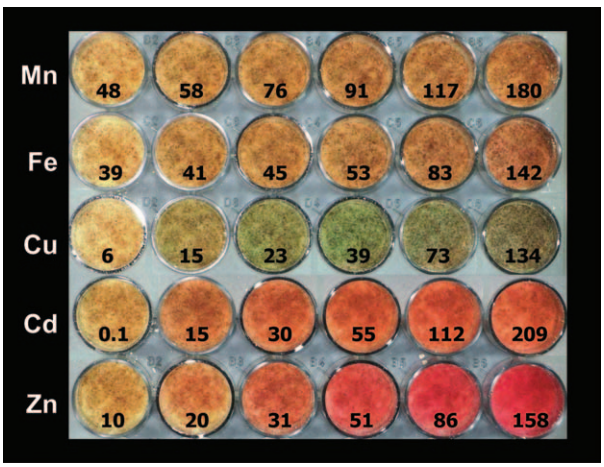

Fig. 4. Effect of different metals on the development of red color due to DTZ staining in whole bread wheat flour (*Triticum aestivum*, cv. BDME-10). The seeds of BDME-10 with low Zn concentration (10 mg kg^{-1}) were incubated in solutions containing increasing amount of Mn, Fe, Cu, Cd or Zn to obtain seeds with different metal concentrations as indicated. All metals were applied as sulfate salts. Following incubation for 20 h in solutions with different metals, seeds were dried at 45°C, ground and treated for DTZ staining.

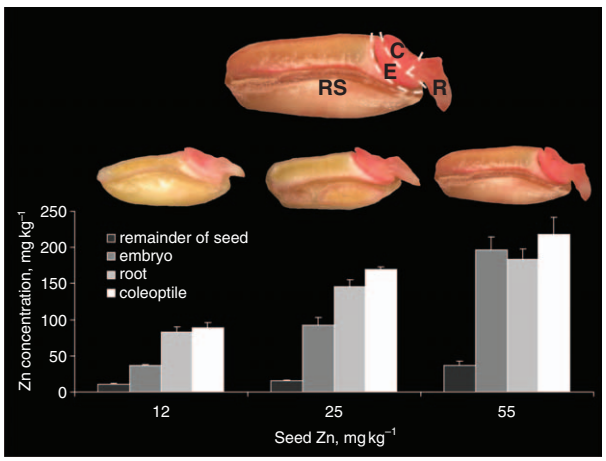

Fig. 6. Concentrations of Zn in different seed parts and DTZ staining of seeds after germination on Petri dishes. Excision of coleoptile (C), root (R), embryo (E) and remainder of seeds (RS) for ICP-OES Zn analysis is shown at the top. The wheat cultivars used were one durum wheat cultivar (*Triticum durum*, cv. Selcuklu) with 12 mg Zn kg^{-1} and one bread wheat cultivar (*Triticum aestivum*, cv. Balatilla) with 25 or 55 mg Zn kg^{-1}. Vertical bars represent ± SD of three independent replications.

Zn concentration. The Zn concentrations of the roots and coleoptile after 48 h of germination were 83 and 89 mg kg^{-1} for the seeds with 12 mg Zn kg^{-1}, and 184 and 218 mg kg^{-1} for the seeds with 55 mg Zn kg^{-1}. Compared to the remainder of the seed (mostly endosperm), the Zn concentrations in the coleoptiles were approximately nine-fold (from 10 to 89 mg kg^{-1}) and six-fold (from 37 to 218 mg kg^{-1}) higher in seeds containing 12 and 55 mg Zn kg^{-1} respectively (Fig. 6).

Staining of whole grain flour derived from field-grown seeds with different Zn concentrations showed an increase in the intensity of red color formation with increasing Zn concentration in the flour (Fig. 7). The dark-reddish patches on the surface of flour samples in Fig. 7 indicate pieces of Zn-rich embryo and aleurone that could not be ground sufficiently finely.

To study the relationship between seed Zn concentration and intensity of the red color produced by the Zn–DTZ complex, the seed Zn concentrations were plotted against the spectral absorbance of the color extracts from the stained flour samples. There was a significant relationship between seed Zn concentration and the spectral absorbance of the flour extracts treated with DTZ (Fig. 8).

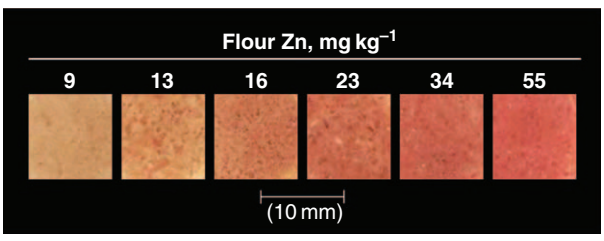

Fig. 7. DTZ staining of whole grain flour of different wheat genotypes. Flour samples from left to right: commercial flour purchased at a supermarket (9 mg Zn kg^{-1}), and whole grain flour of bread wheat cultivars Gerek (13 mg Zn kg^{-1}), Ak-702 (16 mg Zn kg^{-1}) and Balatilla with 23, 34 or 55 mg Zn kg^{-1}. Differences in Zn concentrations in Balatilla bread wheat were obtained by applying different amounts of foliar Zn.

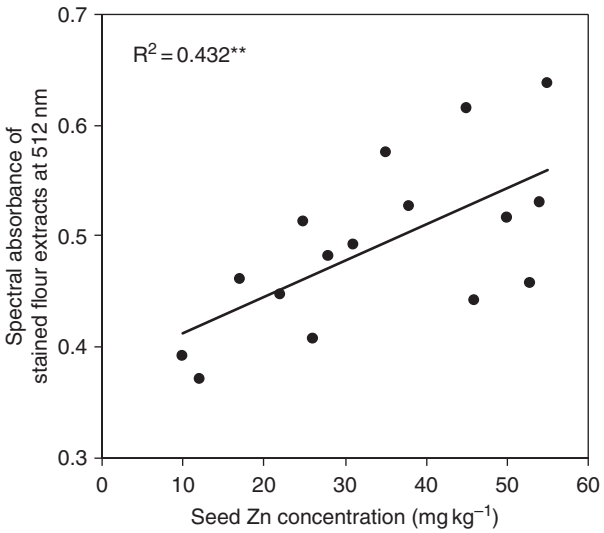

Fig. 8. Relationship between seed Zn concentration and spectral absorbance of methanol extracts of whole wheat flour samples stained with DTZ in two durum (Kunduru and Selcuklu) and two bread wheat (Bagci and Balatilla) cultivars grown in the field and treated (three, six or nine times) or not treated with foliar Zn applications.

Discussion

Foliar Zn applications significantly increased grain Zn concentrations of wheat (Fig. 1), indicating high mobility of Zn within plants. In field experiments conducted on Zn-deficient soils in Central Anatolia, foliar application of Zn fertilizers enhanced seed Zn concentration by a factor of 3 or more when combined with soil Zn application (Yilmaz et al., 1997). A similar observation has been made in wheat seedlings under controlled conditions in short-term experiments using radiolabeled Zn (^{65}Zn). Under Zn-deficient conditions, about 40% of the total absorbed Zn was translocated from the treated leaf into roots and other parts of shoots, whereas in the Zn-adequate plants, this proportion was nearly 25% (Haslett et al., 2001; Erenoglu et al., 2002). In a pot experiment, under greenhouse conditions, up to 77% of the total Zn in whole plant tissue was deposited in the grains of wheat plants treated with Zn (Grewal and Graham, 1999). These results suggest that soil and foliar application of Zn fertilizers is a promising short-term approach to improve Zn concentrations in seeds. In view of the fact that Zn deficiency is a global human nutritional problem, a rapid increase in seed Zn concentration by the use of Zn fertilizers on large areas will greatly contribute to the alleviation of Zn deficiency-related health problems in the developing world (Hotz and Braun, 2004).

The highest Zn accumulation during seed development was found at the early stage of seed formation, e.g. the early milk stage (Fig. 1), suggesting that applying foliar Zn during the late growth stages of wheat (e.g. booting stage) could be an effective way of increasing seed Zn concentration. Possibly, high Zn accumulation during early seed development is related to protein synthesis. There are several reports showing that protein synthesis in seeds is particularly high during early seed development in wheat (Greene, 1983; Martre et al., 2003). Zn is the most critical micronutrient affecting protein synthesis in plants (Cakmak et al., 1989; Obata et al., 1999). There are more than 300 enzymes that require Zn for structural integrity and biological function (Marschner, 1995). As reviewed by Cakmak et al.

(2004), several studies are available showing that the concentrations of Zn and protein in seeds are positively correlated. In a preliminary study, it was found that the genes affecting protein content in wheat seeds are closely related to the genes affecting high Zn accumulation in seeds (D Assaf et al., unpublished results).

Staining of the seeds with DTZ demonstrated that Zn is predominantly localized in the embryo and aleurone (Fig. 2). The embryo and aleurone parts of seeds are also known to be rich in protein and phytate (Lott and Spitzer, 1980; Welch and Graham, 1999; Lin et al., 2005). It seems likely that a high concentration of protein and phytate in these seed parts is a sink for Zn. Ehret (1985) showed that Zn is particularly concentrated in the embryo fraction: in the whole wheat grain, Zn and protein concentrations were 27 mg kg^{-1} and 14.2%, whereas in the seed embryo of the same wheat cultivar, these values were 226 mg kg^{-1} and 42%, respectively. Possibly, enhancements of protein concentration in seeds could result in corresponding increases in seed Zn concentration. A bold policy would call for improving seed Zn concentration by affecting seed N or protein concentrations (e.g. late N applications to soil and/or via foliar applications).

Previously, it has been shown that Zn is localized in the protein bodies of the wheat embryo, with concentrations of up to 600 mg kg^{-1} (Mazzolini et al., 1985). The endosperm of many cereal grains is low in phytate (Lott et al., 1995), and this is in accordance with the low Zn concentration of the endosperm, as found in the present study by using the DTZ staining method (Figs 2, 3 and 5). One plausible explanation for why cereals generally have much lower Zn concentrations in seeds when compared to the seeds of legumes is possibly related to their lower protein concentrations. Currently, studies by our group are in progress investigating the relationship between Zn and protein concentration in cereal and legume seeds.

As presented in Figs 5 and 6, there was an intensive retranslocation of Zn from the Zn pools (e.g. embryo and aleurone) into newly developed roots and coleoptiles. Such high remobilization of Zn into roots and shoots during seed germination (over 12 days) was also shown by Moussavi-Nik et al. (1997). In the study conducted by Moussavi-Nik et al. (1997), Zn concentrations of roots and shoots were not presented. In the present study, we found that Zn concentrations were particularly high in the newly emerged root tips and coleoptile, and reached levels as high as 218 mg kg^{-1} (Fig. 6). Even in the case of seeds with low inherent Zn concentrations (e.g. 12 mg kg^{-1}), up to 89 mg Zn kg^{-1} was found in the coleoptile and roots after 48 h of germination. These results provide strong evidence that Zn is highly mobile during seed germination. However, the possibility should be kept in mind that Zn can also be released (leaked) from seeds during germination, and the released Zn can be re-absorbed by the roots and coleoptile (Powell and Matthews, 1981). However, this possibility appears unlikely, because the duration of the experiment was too short for there to be such high accumulation of Zn in the newly developed organs. In addition, neither the roots (radicle) nor the coleoptile were always in contact with the germination medium.

High Zn concentrations in newly developed roots and leaves (coleoptile) during seed germination indicate that a large amount of Zn is needed in highly metabolically active differentiating cells. In such very actively growing root and shoot meristematic tissues, Zn is most likely utilized in protein synthesis, membrane structure and functions, gene expression and oxidative stress tolerance (Cakmak, 2000). It is therefore not surprising that seeds with low Zn contents show poor seedling vigor and seedling establishment (Welch, 1986, 1999). High Zn concentrations in seeds appear to be critically important in the protection of germinating seeds and developing seedlings from infection by soil-borne pathogens, and in increasing tolerance to different environmental stress factors such as drought, extreme temperatures and salinity. In field and pot experiments, it has been shown that plants derived from seeds with high Zn concentrations perform well and produce high yields (Rengel and Graham, 1995; Yilmaz et al., 1998). In good agreement with these observations, Welch (1999) reported that during the reproductive development of plants, Zn should be provided to seeds at levels that exceed the optimum level for the crop itself to achieve optimum seed viability and seedling vigor, especially when such seeds are sown under environmentally stressful conditions.

The DTZ staining method applied in the present study is a rapid qualitative method for estimation of seed Zn concentration and ranking genotypes for their seed Zn concentration. There was a close relationship between Zn concentrations in the different parts of the germinating seeds measured by ICP-OES and the visual intensity of the red color due to the Zn–DTZ complex (Fig. 6). In addition, flour Zn concentrations were also well correlated with the intensity of the red color (Fig. 7). The staining method was further validated by quantitative analysis of extracts of flour samples stained with DTZ. There was a significant correlation ($P < 0.01$) between Zn concentration and spectral absorbance of extracts, indicating more intensive red color formation in samples with higher Zn concentrations (Fig. 8). Among the metals tested, only Cd can interfere with this method (Fig. 4). Similarly, Pielichowska and Wierzbicka (2004)

also showed that DTZ can react with Cd and form dark red Cd–DTZ complexes in a Cd-hyperaccumulator plant. However, this interference takes place only at extremely high Cd concentrations in seed samples (over 12 mg Cd kg^{-1}), which are very unlikely to occur in seeds of field-grown plants. For example, the Codex Alimentaris Commission suggested a maximum level of 0.2 mg Cd kg^{-1} in crop grains (cited in Hart et al., 2005).

The results of the present study show that Zn is highly mobile both in vegetative tissues and in germinating seeds. There was a large accumulation of Zn in the newly developing coleoptile and radicle, suggesting that Zn is required in the actively growing parts of plants. Similarly, in shoot meristematic tissues of rice plants, the Zn concentration was at least 10-fold higher than in the mature leaves, in order to maintain a high rate of protein synthesis (Kitagishi and Obata, 1986). The DTZ staining method demonstrated that seed Zn mostly accumulates in the aleurone and embryo, which might be associated with high levels of Zn-binding compounds, such as proteins and phytate. The DTZ method is also useful in studying the concentration, localization and mobilization of Zn in seeds, and can be applied as a rapid method for ranking genotypes for seed Zn concentrations. Although not proposed as a robust screening method for Zn, DTZ staining can be useful, at least for distinguishing seed or flour samples with Zn concentrations lower than 20–25 mg kg^{-1}, due to very slight red color production at these Zn concentrations (Figs 4 and 7). When working with flour samples, special care should be taken to use samples with a similar matrix color, in order to minimize possible interference with the stain color by the background color of the sample (e.g. grayish and brownish flour color in some tetraploid and wild wheats).

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