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Is grain zinc concentration in wheat limited by source?

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Abstract

Increasing zinc (Zn) concentration in wheat grain is important to human health. In order to clarify the source limitations of grain Zn concentration, ears of winter wheat were grown in detached ear culture under different Zn supply levels (0, 30, 60, 90 and 120 μmol l^{-1}) and sucrose supply levels (2%, 4% and 6%). To investigate sink limitations, some ears were subjected to partial spikelet removal. The result showed that wheat grain yield did not change; however, the concentration of Zn, iron (Fe), manganese (Mn), and protein in grain was significantly (p<0.05) increased by Zn application. At 90 μ mol l^{-1} Zn supply, grain Zn concentration reached a maximum of 100 mg kg^{-1}; compared to the no Zn treatment, this represented 5.5 to 9.8-fold and 5.7 to 8.4-fold increase, for superior grain and inferior grain, respectively. With the increase in sucrose supply, grain weight, as well as the content of micronutrients and protein significantly (p<0.05) increased, while the concentrations of these significantly (p<0.05) decreased. The partial spikelet removal treatment, along with increasing single grain weight, also increased grain Zn concentration and grain Zn content by 24.0 to 33.4%, 4.1 to 30.8%, and 34.7 to 75.0%, respectively. The concentration and content of Fe, Mn, and protein in wheat grain also increased. These results indicated that Zn concentration in wheat grain was mainly restricted by the source, and the concentration of Zn, Fe, and protein may be increased simultaneously in wheat grain in certain source supply ranges.

Keywords: wheat (*Triticum aestivum* L.); zinc concentration; source and sink manipulation; ear culture.
Abbreviations: Zn_zinc; Fe-iron; Mn_manganese; N-nitrogen; P_phosphorus; K-potassium; AAS_atomic absorption spectrometer; DTPA_diethylene triamine pentacetate acid; TEA_triethanolamine.

Introduction

Zinc (Zn) is one of the most important micronutrients for human health, being involved in many physiological and biochemical processes (Welch and Graham, 2004). It is well documented that Zn deficiency impairs the immune system and increases the incidence of infectious diseases such as diarrhea and pneumonia, especially in the developing world (Walker and Black, 2007). The Zn supply for people in the developing world comes mainly from cereal crops. Wheat is a highly consumed cereal crop (McKevith, 2004). The improvement of Zn concentration in wheat grain to provide better nutrition for humans has been an important task of international agricultural research. In China, annual consumption of wheat is more than 100 million tons, and the mean yield was 4948.5 kg ha^{-1} in 2013. In conventional fields, no Zn fertilizer is generally applied. In recent years, some reports have shown that Zn concentration in wheat grain may be increased by application of fertilizers (Yilmaz et al., 1997; Kalayci et al., 1999), conventional plant breeding (Graham et al., 1998; Welch and Graham, 2004), and genetic engineering techniques (Uauy et al., 2006). However, the regulatory mechanisms of Zn concentration in wheat grain are poorly understood. Some studies showed that applying Zn fertilizer increased grain Zn concentration 1.5 to 3.5 fold, suggesting that the grain Zn concentration was limited by source supply (Yilmaz et al., 1997; Kalayci et al., 1999). However, Pearson et al. (1996) reported that Zn-deficient wheat grain is not a strong sink for Zn, and that Zn loading into the grain was limited at high Zn concentration in the nutrient solution. Meanwhile for rice grain, Jiang et al. (2008) showed that increasing Zn supply increased Zn concentration in polished rice grains (endosperm) from 9 to 37 mg kg^{-1} but remained three to five times lower than that in the bran, such that there seemed to be a barrier for Zn transport between bran and endosperm. In summary, the extent of source or sink limitation of grain Zn concentration needs to be further studied. The correlation of micronutrient concentration to grain yield and grain protein concentration has been continually investigated all along. Some reports have shown that grain Zn concentration correlated negatively with grain yield, and there was a significant decreasing trend in grain Zn concentration with the year of variety release (Pleijel and Danielsson, 2009; Ficco et al., 2009). There is also a report that the decrease in mineral concentration over the past 120 years occurs primarily in the soft white wheat market class, whereas in the hard red market class it has remained largely constant over time (Murphy et al., 2008). Using the abundant variation present in wheat cultivars, it should be possible to improve mineral concentrations in modern cultivars without negatively affecting yield (Murphy et al., 2008). Otherwise, micronutrient-rich cultivars can also be higher yielding than less micronutrient-rich cultivars, especially when grown on soils with low micronutrient availability (Graham et al., 2001). So, there is a contradictory view on the relationship of minerals and grain yield. The objectives of this study were to investigate the extent of source limitation of grain Zn concentration in wheat by culturing ears under different treatments including Zn supply level and sucrose supply level, and to investigate the sink limitation by partial spikelet removal. Additionally, it aimed to analyze the relationships among the concentrations

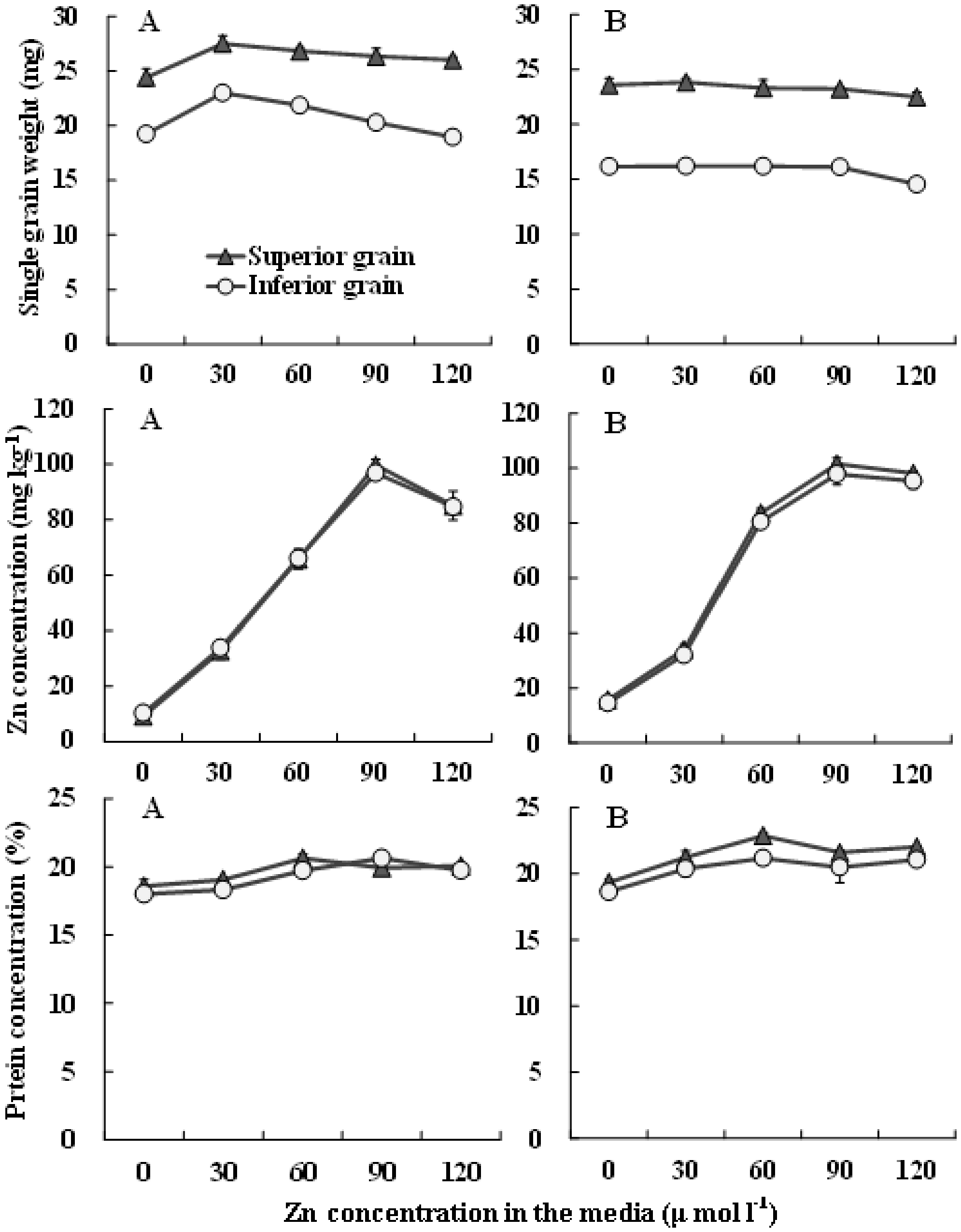

Fig 1. Effect of Zn supply level in the culture media on final grain weight, Zn concentration, and protein concentration of superior grain and inferior grain in JM22 (A) and SM15 (B).

and contents of Zn, Fe, Mn, and protein in wheat grain, with grain yield. These experiments will provide guidance for agronomic practices aimed at improving micronutrient quality of wheat grain.

Results

Effect of Zn supply on grain weight, Zn, Fe, Mn, and protein accumulation

In this study, 0 to 120 μmol l^{-1} Zn supply had no significant effect on grain yield in JM22 and SM15 (Table 1). The single grain weight in JM22 increased as the Zn level increased, but there was no significant difference among 30, 60, and 90 μmol l^{-1}Zn levels. Compared to the 30 μmol l^{-1} Zn supply, the 120 μmol l^{-1}Zn supply decreased single grain weight in the two cultivars, and the decrease was higher in SM15 (6.5%) than in JM22 (4.3%). The responses of superior grain weight and inferior grain weight to Zn supply levels were similar to those of mean single grain weight in the two cultivars; however, the dry weight of superior grain was higher than that of inferior grain (Fig. 1). The concentration and content of Zn in grain increased as Zn supply level increased up to the level of 90 μmol l^{-1}Zn supply, and then decreased (Table 1). This showed that grain Zn accumulation was initially restricted by Zn source supply though it may have become restricted by sink capacity as the grain Zn concentration approached 100 mg kg^{-1}. The changes of Zn concentration of superior and inferior grain were similar in different Zn supply levels (Fig. 1). At 90 μmol l^{-1} Zn level, for JM22, the Zn concentration increased 9.8-fold and 8.4-fold in superior and inferior grain compared to 0 μmol l^{-1}Zn level, respectively; for SM15, the Zn concentration increased by 5.5-fold and 5.7-fold in superior and inferior grain, respectively. At the same time, the Zn concentration in the superior grain was higher, 3.01% and 3.82%, than in the inferior grain for JM22 and SM15, respectively. So, the manipulation effect of source (Zn supply) was higher than sink (superior grain vs inferior grain) for grain Zn accumulation.

The concentration and content of Fe, Mn, and protein in grain also increased as Zn supply level increased, and the maximum occurred either at the 60 or 90 μmol l^{-1} Zn level. At different Zn levels, the concentration and content of Zn and protein in grain were higher in SM15 than in JM22, and the concentration and content of Fe and Mn in grain were higher in JM22 than in SM15. The protein concentration in superior grain and inferior grain both reached the maximum at the 60 or 90 μmol l^{-1} Zn level; however, the protein concentration in the superior grain was slightly higher than in the inferior grain in the two cultivars (Fig. 1).

Effect of sucrose supply on grain weight, Zn, Fe, Mn, and protein accumulation at different Zn levels

Single grain weight increased as sucrose level increased at the 30 μmol l^{-1} and 90 μmol l^{-1} Zn supply levels (Table 2). The highest single grain weight occurred at the low Zn supply (30 μmol l^{-1}) for both JM22 and SM15. Grain Zn concentration decreased as sucrose concentration increased, while grain Zn content increased as sucrose concentration increased at the two Zn supply levels (Table 2), which was the result of the increase in grain weight. Grain Zn concentration and content in the two cultivars were higher at high Zn supply than at low Zn supply. Grain Fe concentration also decreased as sucrose concentration increased at low Zn supply, while at high Zn supply, Fe concentration first increased and then decreased, and grain Fe content increased as sucrose increased at the two Zn supply levels. As sucrose concentration increased, grain Mn and protein concentration significantly decreased, while Mn and protein content increased at the two Zn supply levels. There is no significant difference between low and high Zn levels in Fe, Mn, and protein concentration and content for most sucrose treatments in the two cultivars.

Effect of partial spikelets removal on grain weight, Zn, Fe, Mn and protein accumulation

Compared to control, the partial spikelet removal significantly increased single grain weight at both the low (30 μmol l^{-1}) and high (90 μmol l^{-1}) Zn levels (Table 3). At the low Zn level, single grain weight increased by 33.4% and 23.7%, while at the high Zn level, single grain weight increased by 27.1% and 31.3%, for JM22 and SM15, respectively. This showed that the compensatory increase of single grain weight in JM22 was higher at low Zn level, while in SM15 the compensatory increase was higher at high Zn level. Partial spikelet removal significantly increased grain Zn concentration and content in the two cultivars at low and high Zn levels (Table 3). This showed that grain Zn accumulation in these two cultivars was restricted by Zn source supply. Partial spikelet removal significantly increased the concentration of grain Fe and Mn at low Zn level and the content of Fe and Mn at the two Zn levels in the two cultivars. The increase in Fe and Mn content was due to the simultaneous increase in grain weight with Fe and Mn concentration.

There was no significant difference in grain protein concentration between partial spikelet removal and control at the two Zn levels for the two cultivars, while grain protein content all increased in the partial spikelet removal treatment, which was the result of the increase in grain weight.

The correlations of the concentration or content of Zn, Fe, Mn, and protein with grain yield or grain weight, under source and sink manipulation

In this study (Table 4), under the source manipulation (Zn manipulation and sucrose manipulation), the concentration of Zn, Fe, and Mn in grain were positively correlated with the grain protein concentration but weakly and negatively correlated with grain yield. Under sink manipulation (partial spikelet removal), grain Zn concentration was positively correlated with the grain protein concentration and single grain weight, grain Fe concentration was positively correlated with grain protein concentration but weakly and negatively correlated with grain weight, while grain Mn concentration was negatively correlated with grain protein but positively correlated with grain weight. Under the source and sink manipulation, there were positive correlations among Zn, Fe, and protein concentrations and among the content of Zn, Fe, Mn, and protein and grain yield or single grain weight.

Discussion

The source and sink limitation of Zn accumulation in wheat grains

For wheat, increasing Zn supply seems to be an adequate solution for increasing grain Zn concentration (Waters et al., 2009), implying that grain sink strength or translocation to grain are not limiting factors. However, Pearson et al. (1995) found that increasing the concentration of Zn in the culture solution from 10 to 100 μmol l^{-1} did not result in a tenfold increase in grain loading of Zn. Very recently, Wang et al. (2011) found that two barriers of Zn transport into wheat grain may exist: between the stem, rachis and the grain, and the maternal and filiar tissues in the grain. Stomph et al. (2011) also found that although the Zn status of the wheat plant is improved through increased Zn supply, Zn transport into the endosperm becomes relatively more difficult as compared with Zn unloading from the rachis to the seed vascular tissue or the internal transport in the vascular tissue. There appears to be a major internal transport limitation between the vascular tissue and the endosperm (Stomph et al., 2011), not due to the phloem unloading step or the mainly symplastic transport within the crease tissue (Wang and Fisher, 1994), because when the grain crease Zn concentration is raised to very high levels by increasing external Zn supply, the endosperm Zn concentration will not increase correspondingly (Stomph et al., 2011). In this study, when Zn supply was increased threefold, from 30 μmol l^{-1} Zn to 90 μmol l^{-1} Zn, grain Zn concentration and content also showed a threefold increase. At the 90 μmol l^{-1} Zn level, grain Zn concentration increased 9.5-fold and 5.5-fold compared to no Zn supply for JM22 and SM15, respectively, and reached the maximum of 100 mg kg^{-1}. After that, increasing the Zn supply did not increase grain Zn concentration. This showed that grain Zn concentration in wheat is mainly restricted by Zn supply before it reaches 100 mg kg^{-1}. Therefore, increasing Zn supply in the field may significantly increase grain Zn concentration. Increasing sucrose supply at low and high Zn levels increased grain weight; however, grain Zn concentration decreased, which was the dilution result of the increase in grain weight; grain Zn

Table 1. Effect of Zn supply level in the culture media on final grain weight and the concentrations and content of Zn, Fe, Mn, and protein in wheat grain.

Cultivar	Zn supply level (μmol l^{-1})	Single grain weight (mg)	Micronutrient concentration (mg kg^{-1})			Protein concentration (%)	Grain yield (g ear^{-1})	Micronutrient content (μg ear^{-1})			Protein content (mg ear^{-1})
			Zn	Fe	Mn			Zn	Fe	Mn	
JM22	0	25.34±0.27b	9.41±1.03e	69.61±5.35c	33.11±1.64c	18.46±0.33c	1.03±0.04a	9.71±1.45e	71.85±6.24c	34.18±2.01b	190.55±24.78c
	30	26.40±0.34a	32.95±1.89d	81.96±4.22b	42.44±2.73b	18.92±0.27bc	1.08±0.01a	35.59±2.89d	88.52±5.23bc	45.84±3.42a	204.34±18.90b
	60	25.88±0.38a	65.76±3.81c	102.93±8.76a	40.81±3.40b	20.41±0.26a	1.06±0.03a	69.96±4.35c	109.50±9.34a	43.41±3.89a	217.13±34.26a
	90	25.49±0.48ab	99.11±4.52a	92.28±3.93ab	42.73±2.50b	20.06±0.37ab	1.03±0.03a	102.46±5.34a	95.40±4.38b	44.18±3.42a	207.38±19.68b
	120	25.27±0.24b	84.91±5.75b	83.42±2.35b	44.00±3.98a	19.99±0.31abc	1.04±0.02a	87.96±6.98b	86.42±3.67bc	45.58±4.06a	207.08±10.76b
SM15	0	22.97±0.35a	15.47±1.81d	69.31±5.25d	29.78±1.23d	19.18±0.32d	1.03±0.02a	15.93±2.03d	71.39±5.89c	30.67±2.45c	197.55±12.39b
	30	23.33±0.29a	33.59±3.14c	81.64±4.98c	33.28±2.21c	21.02±0.47c	1.04±0.01a	34.93±3.58c	84.91±5.34b	34.61±3.06b	218.61±12.89a
	60	22.51±0.45a	82.75±4.09b	86.09±3.77b	38.22±2.63b	22.38±0.25a	1.01±0.03a	83.96±4.98b	87.35±4.56b	38.78±3.67b	227.07±19.43a
	90	22.45±0.47a	100.74±7.08a	98.48±4.52a	43.06±3.62a	21.28±0.29bc	1.00±0.02a	101.21±9.67a	98.94±5.98a	43.26±3.56a	213.80±20.67a
	120	21.81±0.27b	97.49±5.91a	81.54±3.42c	36.99±2.03b	21.79±0.37ab	0.98±0.02a	95.96±7.45a	80.26±4.77b	36.41±2.47b	214.48±11.13a

Data was shown in Mean±SE (n=3). Values in the same column followed by the same letters are not significantly different according to Duncan's multiple range test (p<0.05) for JM22 or SM15.

Table 2. Effect of sucrose supply level on final grain weight and the concentrations and content of Zn, Fe, Mn, and protein in wheat grain under different Zn supply levels in the culture media.

Cultivar	Zn supply level (μmol l^{-1})	Sucrose supply level (%)	Single grain weight (mg)	Micronutrient concentration (mg kg^{-1})			Protein Concentration (%)	Grain yield (g ear^{-1})	Micronutrient content (μg ear^{-1})			Protein content (mg ear^{-1})
				Zn	Fe	Mn			Zn	Fe	Mn	
JM22	30	2	17.03±0.49c	50.80±2.80d	81.30±5.02b	43.66±2.29a	26.63±1.23a	0.65±0.03c	32.99±2.96e	52.80±5.67c	28.35±3.12c	172.94±12.89c
		4	26.40±0.32b	32.99±2.86e	81.70±4.64b	41.86±2.11a	18.94±0.64b	1.10±0.02b	36.44±3.42e	90.23±4.84b	46.23±2.67b	209.19±22.33b
		6	36.99±0.56a	32.23±2.77e	68.86±6.01c	38.02±1.78b	16.44±0.85c	1.48±0.01a	47.67±3.01d	101.84±6.57a	56.23±3.21a	243.15±11.78a
	90	2	15.36±0.34c	114.50±4.99a	80.04±5.88b	44.41±2.09a	26.18±0.79a	0.56±0.05c	64.64±5.23c	45.19±6.87d	25.07±2.78c	147.80±13.28c
		4	25.49±0.32b	99.07±5.87b	89.92±3.47a	43.17±3.19a	20.28±0.61b	1.03±0.01b	102.42±7.45b	92.96±4.79b	44.63±3.85b	209.65±17.56b
		6	34.65±0.42a	76.93±4.76c	69.82±4.92c	39.41±3.90b	16.74±0.80c	1.47±0.03a	113.13±5.31a	102.67±5.31a	57.95±4.23a	246.17±21.43a
SM15	30	2	13.26±0.40c	43.39±2.81c	81.71±4.80b	37.33±2.12b	28.06±0.73a	0.58±0.06c	25.19±3.12f	47.43±4.99d	21.67±2.76d	162.88±14.68b
		4	23.33±0.23b	33.59±3.14d	80.54±5.20b	33.32±2.41c	21.02±0.67b	1.07±0.03b	35.86±3.34de	85.99±5.84b	35.58±2.89c	224.43±10.49a
		6	28.75±0.56a	32.54±2.03d	71.71±3.54c	38.57±2.63ab	16.91±0.46c	1.34±0.07a	43.57±2.31d	96.01±3.77a	51.64±3.11a	226.40±21.46a
	90	2	14.35±0.56c	105.50±5.32a	88.27±4.10b	40.63±3.79a	26.65±0.59a	0.62±0.05c	65.54±5.46c	54.84±4.68cd	25.24±5.23d	165.56±12.98b
		4	22.45±0.43b	102.42±6.52a	98.69±3.52a	43.10±1.72a	21.28±0.37b	1.00±0.02b	102.42±7.09a	98.69±4.02a	43.10±2.76b	212.80±24.65a
		6	27.50±0.61a	69.03±4.23b	63.35±2.94d	34.82±2.67c	17.27±0.42c	1.21±0.06a	83.51±5.48b	76.64±3.45c	42.13±3.15b	208.93±11.57a

Data was shown in Mean±SE (n=3). Values in the same column followed by the same letters are not significantly different according to Duncan's multiple range test (p<0.05) for JM22 or SM15.

content increased as sucrose level increased, which showed that grain Zn absorption was increased, and the absorption was higher at the high Zn level than at the low Zn level. Our field experiment had shown that partial spikelet removal significantly increased grain Zn, Fe, and Mn concentration (Zhang et al., 2012). In this study, partial spikelet removal at low and high Zn supply levels in the detached ear culture showed similar results, also significantly increasing grain Zn concentration in the two cultivars. The partial spikelet removal reduced grain number and total sink size, serving to relatively increase the source supply to the remaining grains. These results showed that grain Zn concentration in wheat production was mainly restricted by source supply. In this study, grain Zn concentration in the two popular cultivars was increased to 100 mg kg^{-1} by increasing Zn supply, while the grain Zn concentration in the field for JM22 and SM15 were 35.79 and 37.41 mg kg^{-1}, respectively. This showed that there is huge potential to increase grain Zn concentration by increasing Zn source supply. In production practice, applying Zn fertilizer may be a good way to improve grain Zn concentration; at the same time, it can also increase Fe, Mn, and protein concentration.

The correlations of the concentration or content of Zn, Fe, Mn, and protein with grain yield or grain weight

In the field experiment, some studies have shown that applying Zn fertilizer not only improved grain yield, but also increased grain Zn concentration (Yilmaz et al., 1997; Kalayci et al., 1999). Grain Fe and Zn concentrations were correlated with yield potential, and the yield did not dilute Fe and Zn concentrations (Calderini and Ortiz-Monasterio, 2003; Welch and Graham, 2004). However, other reports showed that yield diluted grain micronutrient concentration (Pleijel and Danielsson, 2009; Ficco et al., 2009), but Fe and Zn concentrations were positively correlated with the grain protein concentration in wheat (Cakmak et al., 2004; Peleg et al., 2008). In this study, increasing the Zn level in the culture solution significantly increased grain Zn concentration but had no significant effect on grain yield. Grain Fe, Mn, and protein concentration showed similar changes as Zn concentration. Under the source manipulation, the concentrations of Zn, Fe, and Mn in grains were positively correlated with the grain protein concentration but weakly and negatively correlated with grain yield. Conversely, under the sink manipulation, the concentrations of grain Zn and Fe were positively correlated with grain protein concentration, and the concentrations of grain Zn and Mn were positively correlated with grain weight. There were also positive correlations among Zn, Fe and Mn concentrations or contents. These results showed that grain yield diluted Fe and Zn concentration to some extent, but grain Zn content and grain yield can be simultaneously increased, and that the concentrations of grain Fe, Zn, and protein can be improved simultaneously. Very recently, new wild emmer wheat accessions have been identified showing simultaneously both very high concentrations of Zn (up to 139 mg kg^{-1}), Fe (up to 88 mg kg^{-1}), and protein (up to 380 mg kg^{-1}) in seeds, along with high tolerance to drought stress, and in Zn deficient soil (Peleg et al., 2008). In grain, Zn is most probably used for protein synthesis, membrane function, cell elongation, and tolerance to environmental stresses (Cakmak, 2000). So, the phloem transport of N and mineral micronutrients may be directly related (Waters and Sankaran, 2011). A RNAi line with delayed leaf senescence had lower remobilization of N, Fe, and Zn and lower grain concentration of these nutrients (Waters et al., 2009), suggesting possible co-transport mechanisms. Another study found the co-localication of Mn, Cu, Zn, and P in wheat grain (Wang et al., 2012), this may imply that the translocation and accumulation of Mn, Cu, Zn, and P in wheat grain are related to each other. These findings are useful for wheat biofortification programs in the future. In summary, increasing Zn supply is a good way to increase grain Zn concentration up to 100 mg kg^{-1} while simultaneously increasing grain Fe, Mn, and protein concentration. On the other hand, since the two cultivars used here had different responses to the environmental Zn supply, further investigation is needed into the mechanism of manipulation of Zn concentration and the reason for variation among the cultivars.

Materials and Methods

Plant materials and cultivation

Two high yield soft white winter wheat (*Triticum aestivum*, L.) cultivars, Jimai 22 (JM22) and Shimai 15 (SM15), were planted on 15 October 2011 at Wuqiao Experiment Station of China Agricultural University at Cangzhou, Hebei province, China. The soil nutrient concentrations in this experimental site are shown in Table 5. Soil organic matter was analyzed using the Walkley-Black method (Walkley and Black, 1934). Soil available nitrogen (N), phosphorus (P), and exchangeable potassium (K) were analyzed by extracting 5.0 g of soil with 50 ml of 2 M KCl, 100 ml of 0.5 M $NaHCO_3$, and 50 ml of 1.0 M NH_4OAc, respectively (Page et al., 1982). DTPA-extractable soil Zn, Fe, and Mn (DTPA-Zn, -Fe, -Mn) were obtained by extracting 10 g soil (<2 mm) with a 20 ml of 0.005 M DTPA + 0.01 M $CaCl_2$ + 0.1 M TEA (triethanolamine) solution (Lindsay and Norvell, 1978). After 2 h of continuous shaking at room temperature, the soil suspension was centrifuged and filtered through a 0.45 mm membrane. Zn, Fe, and Mn in the extract were then analyzed with an atomic absorption spectrometer (AAS). The cultivars were grown in triplicate plots of uniform sizes – 18 m^2 (6×3m). Irrigation was applied at the jointing and flowering stages at the rate of 750 m^3 ha^{-1}. Fertilizer was applied before planting to provide 157.5 kg ha^{-1} N (as urea), 138 kg ha^{-1} P_2O_5 (as ammonium monoacid phosphate), and 120 kg ha^{-1} K_2O (as potassium sulfate). No fertilizer was applied during the growing season. Phoxim and Carbendazol as a seed treatment were used to prevent subterranean pest-insect and pathogenic bacteria, and Dimethoate was used to prevent aphids before anthesis. Primary culms flowering on the same day with similar plant height and ear length were tagged for subsequent detachment.

Detached ear culture

Detached ears were cultured according to the method of Singh and Jenner (1983) and Lee et al. (1989), with slight modifications. Five days after flowering, the tagged primary culms at anthesis were detached below the peduncular node, sterilized with sodium hypochlorite solution (0.5% available chlorine), and then recut 2 cm under sterile distilled H_2O. The flag leaf blade was removed. Detached ears were inserted through cotton plugs into sterile jars containing 300 ml of sterile culture media. The basic culture media was prepared according to Lee et al. (1989) except for varying $ZnSO_4 \cdot 7H_2O$ and sucrose concentration. In the Zn supply level experiment, the concentrations of Zn in the solution were 0, 30, 60, 90, and 120 μmol l^{-1}. The sucrose supply level experiment was conducted at 30 and 90 μmol l^{-1} Zn levels, the concentration of sucrose in the solution was 2%, 4%, and 6%. The sink regulation experiment also was conducted at 30 and 90 μmol l^{-1} Zn levels; for this trial, partial spikelet removal was performed by removing the spikelets of the top half of the ear, while the

Table 3. Effect of sink treatment on final grain weight and the concentrations and content of Zn, Fe, Mn, and protein in wheat grain under different Zn supply levels in the culture media.

Cultivar	Zn supply level (μmol l^{-1})	Sink Treatment	Single grain weight (mg)	Micronutrient concentration (mg kg^{-1})			Protein concentration (%)	Micronutrient content (μg grain^{-1})			Protein content (mg grain^{-1})
				Zn	Fe	Mn		Zn	Fe	Mn	
JM22	30	Half spike removal	36.48±0.47a	44.00±4.21c	89.40±7.20ab	56.60±3.96a	18.39±0.70bc	1.61±0.12c	3.26±0.21a	2.06±0.15a	6.71±0.14a
		Control	27.35±0.57b	33.76±3.32d	84.28±5.60b	45.23±4.07b	17.57±0.60c	0.92±0.10d	2.31±0.17c	1.24±0.08b	4.81±0.21b
	90	Half spike removal	34.19±0.31a	104.29±7.56a	87.34±6.77ab	55.35±3.03a	18.72±0.43a	3.57±0.08a	2.99±0.13a	1.89±0.16a	6.40±0.19a
		Control	26.89±0.51b	98.51±5.41b	95.56±7.59a	44.63±2.36b	18.53±0.52ab	2.65±0.15b	2.57±0.22b	1.20±0.11b	4.98±0.31b
SM15	30	Half spike removal	29.52±0.35a	42.31±3.47c	92.97±6.41b	48.56±4.87a	19.16±0.47a	1.25±0.11c	2.74±0.21a	1.43±0.25a	5.66±0.24a
		Control	23.86±0.37b	32.35±2.42d	81.62±4.87c	33.49±2.12d	19.54±0.36a	0.77±0.13d	1.95±0.15d	0.80±0.09d	4.67±0.18b
	90	Half spike removal	30.38±0.46a	107.09±8.26a	86.30±7.65bc	40.20±3.55c	19.55±0.30a	3.25±0.09a	2.62±0.14b	1.22±0.16b	5.94±0.37a
		Control	23.13±0.28b	102.90±7.80ab	100.67±8.99a	43.30±2.27b	19.56±0.66a	2.38±0.12b	2.33±0.17c	1.00±0.14c	4.52±0.25b

Data was shown in Mean±SE (n=3). Values in the same column followed by the same letters are not significantly different according to Duncan's multiple range test (p<0.05) for JM22 or SM15.

Table 4. Correlations of the concentrations or content of grain micronutrients and protein and grain yield or single grain weight under source and sink manipulation of detached ear culture.

Treatment	Micronutrient and protein concentration or content	Concentration			Content			Grain yield	Single grain weight
		Fe	Mn	Protein	Fe	Mn	Protein		
Zn supply levels, sucrose supply levels	Zn concentration	0.549**	0.631**	0.353				-0.304	
	Fe concentration		0.575**	0.386				-0.358	
	Mn concentration			0.262				-0.238	
	Protein concentration							-0.947**	
	Zn content				0.433*	0.414	0.321	0.188	
	Fe content					0.876**	0.892**	0.813**	
	Mn content						0.844**	0.905**	
	Protein content							0.912**	
Sink manipulation	Zn concentration	0.478	0.07	0.341					0.018
	Fe concentration		0.21	0.203					-0.212
	Mn concentration			-0.488					0.824*
	Protein concentration								-0.341
	Zn content				0.408	0.284	0.393		0.315
	Fe content					0.954**	0.915**		0.928**
	Mn content						0.905**		0.955**
	Protein content								0.975**

* p <0.05; ** p <0.01. Positive value means positive correlation, and negative value means negative correlation.

Table 5. Concentrations of soil nutrients in the study.

Soil depth	Organic matter (mg g^{-1})	Available N (mg kg^{-1})	Available P (mg kg^{-1})	Exchangeable K (mg kg^{-1})	DTPA-Fe (mg kg^{-1})	DTPA-Zn (mg kg^{-1})	DTPA-Mn (mg kg^{-1})
0–20 cm	13.1±0.4	13.2±0.3	23.5±1.0	55.6±0.6	8.4±0.9	2.3±0.2	4.3±0.1
20–40 cm	6.1±0.1	10.2±0.2	6.2±0.3	48.7±0.4	7.9±0.5	1.2±0.1	3.9±0.3

Data was shown in Mean±SE (n=3). DTPA: Diethylene triamine pentacetate acid, it is a chelator.

control was an intact ear. Additional investigation of sink limitation was performed by comparing results for superior vs inferior grain classes as described below. The treatments were arranged as a randomized complete block design with three replications. Each replication included 6 jars, and each jar contained five ears. The jars containing the detached ears were placed in a circular water bath at 2 °C in a growth chamber with 16 h of light (high output, cool-white fluorescent tubes providing about 160 μmol m^{-2} s^{-1} light at ear-level), at a constant air temperature of 24 °C. The culture media in the jar was renewed every five days. At maturity, yield components, and the concentrations and contents of Zn, Fe, Mn, and protein were all measured.

Sample analysis

At maturity, grain samples from each replicate were dried at 60 °C for 48 h, then weighed, counted (grain number per ear), and milled in a stainless steel grinder. Milled samples were stored in a desiccator until Zn, Fe, Mn, and protein concentration were analyzed. For an additional comparison of sink-limitation effects, some of the grains from the Zn level experiment were separated into classes of "superior grain" (the basal grains including the first and second grain in a spikelet) and "inferior grain" (the middle grains including the third and fourth grain in a spikelet). The grain weight and the concentrations of Zn and protein for these superior grain and inferior grain classes were then measured and compared. Grain Zn, Fe, and Mn concentrations were analyzed using AAS (AA300, Perkin Elmer, Shelton, USA) (Calderini and Ortiz-Monasterio, 2003). A 0.5 g dry sample was digested with $HClO_4$–HNO_3 till becoming a transparent solution, and then the digested solution was analyzed with AAS. Mineral standards were run for each analysis to ensure the accuracy of estimation. Grain micronutrient contents were obtained by multiplying micronutrient concentrations by grain weight per ear or per grain. The nitrogen concentration of grain was measured on 200 mg of dry powder, using the standard macro-Kjeldahl procedure (Nitrogen Analysis System, Büchi, Switzerland), and the protein concentration was calculated from the nitrogen concentration by multiplying by a factor of 5.83. Three runs were made for each sample, and the mean was used for statistical analysis. Grain protein content was obtained by multiplying protein concentration by grain weight per ear or per grain.

Statistical analysis

Data were subjected to ANOVA using PROC GLM (SAS 8.1). Duncan's multiple range test was used to compare the mean differences among the treatments at the 5% probability level. Correlation analysis was performed (SPSS 15.0) to explore the correlations of the concentrations or content of Zn, Fe, Mn and protein to grain yield or single grain weight, and the correlations among the concentrations or content of micronutrients and protein.

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