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The Effectiveness of Foliar Applications of Zinc and Biostimulants to Increase Zinc Concentration and Bioavailability of Wheat Grain

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Abstract: Increasing zinc (Zn) concentration in wheat grain is an important global challenge due to high incidence of Zn deficiency in human populations. In this study, a two-year field experiment was conducted to investigate the effects of foliar $ZnSO_4$ combined with various biostimulants (fulvic acid (FA), seaweed extract (SE), amino acids (AA), and microbial incubates (MI)) on Zn concentration and bioavailability in wheat grain under different soil nitrogen (N) levels (0, 120, and 240 kg N/ha). Grain Zn concentration and bioavailability were significantly enhanced by foliar Zn plus various biostimulants and soil N supply. Compared to foliar Zn alone, foliar Zn + FA resulted in 16% increase in grain Zn, mainly from insoluble Zn increases, while foliar Zn + AA caused 11% increase in grain Zn, mainly from soluble (at N0) and insoluble Zn increases (at N120). Foliar Zn + FA and Zn + AA generally resulted in higher Zn bioavailability than foliar Zn alone. Additionally, N concentration and Fe concentration and bioavailability in grain were enhanced with foliar Zn + AA and soil N application. Thus, foliar $ZnSO_4$ plus FA and AA under optimal soil N rate (120 kg N/ha) can be an effective and economically friendly approach for achieving agronomic biofortification.

Keywords: zinc; biostimulants; foliar application; wheat grain; Zn forms

1. Introduction

Zinc deficiency is the most prevalent micronutrient deficiency in human beings, negatively affecting at least one-third of the world population [1]. Insufficient dietary intake of Zn is the major reason for the widespread prevalence of Zn deficiency in humans [2]. Wheat, an important food source for two-thirds of the world population, provides up to 70% of the daily calorie intake in rural areas [3] and more than 20% of the daily Zn intake in China [4]. However, wheat is inherently low in Zn, and there is strong evidence that rising concentrations of atmospheric CO_2 leads to reduced levels of Zn in C-3 crops, including wheat [5,6]. Thus, improving the concentration of Zn in wheat grain has become a high-priority research area.

Agronomic biofortification via Zn fertilization, especially by foliar Zn application, is thought to be the most useful, cost-effective, and applicable solution for achieving Zn biofortification in wheat [2]. With foliar application, Zn is absorbed by the leaf epidermis and easily transferred to the developing grain through the phloem [7]. Thus, foliar application of Zn is more effective in

increasing grain Zn concentration than soil application [8,9]. However, to avoid scorching of leaves and environmental hazards, Zn must be applied to leaves at low doses, typically as $ZnSO_4$ at the rate of 1–2 kg ha^{-1} with solute concentration of 0.3%–0.5%. Even so, only a small portion (<6%) of applied Zn is taken up by the plant [10]. Thus, optimizing the efficacy of the foliar-applied Zn and achieving target level of grain Zn while avoiding plant damage and environmental hazards remains a challenge.

One option to improve the efficiency of foliar Zn formulations is the addition of plant biostimulants [11]. In the past decades, the use of biostimulants in agriculture has grown dramatically because they can (i) enhance plant growth and development when applied in small quantities; and (ii) improve the efficiency of plant nutrients, as measured by either improved nutrient uptake or reduced nutrient losses to the environment [11,12]. It is therefore speculated that the addition of biostimulants to foliar Zn applications may help to increase the potential of biofortification with Zn and thereby alleviate human Zn malnutrition. There are four major categories of compounds, namely microbial inoculants, fulvic acid, amino acids, and seaweed extracts, that can potentially act as biostimulant [11]. Most of the studies assessing the effect of these biostimulants have focused on promoting plant growth and productivity [13]; only a few studies have investigated the impact of foliar biostimulants on the uptake of micronutrients in plants, including Zn. For example, Tian et al. [14] reported that foliar spray microbial inoculants increased the movement of leaf-applied Zn in sunflower. Koksal et al. [15] showed that leaf-applied amino acid chelate in pear increased leaf Zn content. In addition, Rauthan and Schnitzer [16] suggest that cucumber treated with fulvic acid enhances Zn content in shoot. However, those studies were conducted on horticultural plants; no information is available on how the combination of various biostimulants and Zn through foliar applications affects the concentration of Zn in wheat grain.

Zinc is present in plants in water-soluble and -insoluble forms [17]. Previous studies indicate that the effect of fertilizer applications on Zn concentration can be explained by the changes in different forms of Zn. For example, in a field study conducted by Li et al. [18], foliar Zn application mainly increased water-soluble Zn (Zn-Nicotianamine synthase (NAs)) in flour and water-insoluble Zn in grain and bran, which thereby increased total Zn concentrations. In addition, the positive effect of high soil N application on grain Zn concentrations was attributed to an increase in water-insoluble Zn, probably in the form of water-insoluble proteins [19]. Accordingly, it is critical to determine the different forms of Zn in grain in order to explain the mechanism of the effect of foliar biostimulants in combination with Zn application on grain Zn concentration. Furthermore, optimizing N management by reducing soil N input has become a trend to protect the environment [20]. The question was whether changes in N management will affect the Zn concentration and form (water-soluble or -insoluble) in wheat grain in response to combined foliar application of biostimulants and Zn.

Apart from inadequate dietary intake of Zn, low bioavailability of Zn is another common causative factor for Zn deficiency. Phytic acid (PA), present in cereal grains in relatively large quantities, is a potent inhibitor of Zn bioavailability in food [21]. In contrast, high concentrations of protein and amino acids in grain results in higher bioavailability of Zn in the diet [22]. Recently, the PA-to-Zn molar ratio and total daily-absorbed Zn (TAZ) (mg Zn d^{-1}), which was calculated by a trivariate model of Zn absorption, were both used to estimated Zn bioavailability [23]. Studies have shown that soil N and foliar Zn application are effective in increasing Zn bioavailability [24,25], while little is known about their interactions with foliar biostimulants in increasing Zn bioavailability.

In this field research study, which was repeated over 2 years, we investigated (1) the effects of four biostimulants combined with foliar Zn application on the concentrations of Zn, Fe, Mn, N, P, and K in wheat grain under different soil N rates; (2) the responses of different forms of Zn in wheat grain to the above foliar applications and different rates of soil N; and (3) the PA concentration and bioavailability of Zn as affected by foliar biostimulants and Zn application.

2. Material and Methods

2.1. Field Location

The experiment was conducted at the Experimental Farm of the Northwest A&F University, Yangling, Shaanxi Province, China, during the winter seasons (October to June) of 2014–2015 and 2015–2016. The elevation of the experimental site is 525 m and the climate is semi-humid. The average annual temperature of this region is 13 °C, with a mean annual rainfall of 600 mm. The soil at the experimental site is classified as Eum-Orthic Anthrosol (manual loessial soil, formed in this region) and has the following characteristics: pH 7.98 (1:5 of soil/water), 13.9 g kg^{-1} organic matter, 12.3 mg kg^{-1} Olsen-P, 11.4 mg kg^{-1} NO_3-N, 3.03 mg kg^{-1} NH_4-N, 140 mg kg^{-1} available K, 65.1 g kg^{-1} $CaCO_3$, and 0.75 mg kg^{-1} DTPA-Zn. The wheat cultivar used was Xiaoyan 22 (*Triticum aestivum L.*).

2.2. Experimental Design

The experimental design was a split-plot design with four replications. Main plot (6 m × 10 m) treatments consisted of three soil N rates that applied as urea: no soil N application (N0), reduced soil N application rate (120 kg N ha^{-1}, designated N120), and conventional soil N application rate (240 kg N ha^{-1}, designated N240). These plots (10 m × 6 m) have been part of a long-term experiment and received the same three soil N fertilization rates every year since 2002. The sub-plot treatments included: (1) foliar application of distilled water (CK); (2) foliar application of 0.4% (*w*/*v*) $ZnSO_4 \cdot 7H_2O$ (Zn); (3) foliar application of 0.4% (*w*/*v*) $ZnSO_4 \cdot 7H_2O$ plus 0.1% (*w*/*v*) fulvic acid (Zn + FA); (4) foliar application of 0.4% (*w*/*v*) $ZnSO_4 \cdot 7H_2O$ plus 0.1% (*v*/*v*) seaweed extracts (Zn + SE); (5) foliar application of 0.4% (*w*/*v*) $ZnSO_4 \cdot 7H_2O$ plus 0.1% (*w*/*v*) amino acids (Zn + AA); (6) foliar application of 0.4% (*w*/*v*) $ZnSO_4 \cdot 7H_2O$ plus 2.5% (*v*/*v*) microbial inoculants (Zn + MI).

Before sowing, nitrogen fertilizer and phosphate fertilizer (100 kg of P_2O_5/ha) as superphosphate were incorporated into the 0–25 cm soil layer by plow. Foliar applied solutions contained 0.01% (*v*/*v*) Tween 20 as a surfactant and were sprayed two times (7 d intervals) during the early milk stage (29 April–13 May) using a cylinder hand sprayer (330 mL capacity, produced by Youhua Plastic Co., Ltd., Taizhou city, Zhejiang Province, China). For all treatments, a solution volume of 1000 L ha^{-1} was used each time. The spray was performed after sunset to avoid possible leaf damage caused by salts on sunny days and at high day temperatures.

2.3. Wheat Grain Sampling

At harvest, wheat samples were harvested individually whole-plot-wise, manually threshed, and weighed to determine the grain yield. The grains of wheat plants were washed carefully by tap water and deionized water, then dried at 60 °C for 48 h, and ground with a ball mill (TL2020) for nutrient analysis.

2.4. Nutrient Analysis

Whole grain samples were digested with concentrated H_2SO_4-H_2O_2. Phosphorus concentrations in the resulting digested solution were analyzed using a vanadate–molybdate–yellow colorimeter, K concentrations were estimated using flame photometer, and N concentrations were determined by the micro-Kjeldahl procedure. The PA concentration was determined based on the precipitation of ferric phytate and measurement of iron remaining in the solution. The details of the determination were described by Wang et al. [10]. Grain protein concentration was analyzed by AutoAnalyzer (AA3) after digestion with H_2SO_4-H_2O_2.

The total Zn, Fe, and Mn concentrations were measured by atomic absorption spectrometry (AA320CRT, Shanghai, China) after being combusted (at 550 ℃ in a muffle furnace for 6 h), and dissolved in 1:1 (*v*:*v*) HNO_3 solution. Reagent blanks and standard wheat flour GBW 08503c were used as controls. Soluble Zn in grain samples was extracted according to the method described by Li et al. [18] and Eagling et al. [26]. Specifically, approximately 0.1 g of grain was weighed and

extracted in 7 mL of 50 Mm Tris-HCl buffer at pH 7.5 for 18 h with continuous shaking (120 rpm, 37 °C). After this, extracts were centrifuged (21,000 g at 13 °C for 10 min) and filtered. Soluble Zn concentration in the filtrate was determined using atomic absorption spectrometry (AA320CRT; Shanghai Precision & Scientific Instrument Co., Shanghai, China). Insoluble Zn concentration in grain was estimated by the difference between the total and soluble concentration.

To predict Zn bioavailability, the PA to Zn, Fe, or Mn molar ratios in samples was calculated by dividing millimoles of PA with millimoles of Zn, Fe, or Mn, respectively. In addition, using the trivariate model of Zn absorption, total daily-absorbed Zn (TAZ) (mg Zn d^{-1}) in grain was calculated as follows:

$$\mathrm{TAZ} = 0.5 \times 65 \times 100 \times \left\{ A_{MAX} + \mathrm{TDZ} + K_R \times \left(1 + \frac{\mathrm{TDP}}{K_P}\right) - \sqrt{\left[A_{MAX} + \mathrm{TDZ} + K_R \times \left(1 + \frac{\mathrm{TDP}}{K_P}\right)\right]^2 - 4 \times A_{MAX} \times \mathrm{TDZ}} \right\} \quad (1)$$

The parameters A_{MAX} (maximum absorption, 0.091), K_R (equilibrium dissociation constant of the Zn-receptor binding reaction, 0.680), and K_P (equilibrium dissociation constant of the Zn-PA binding reaction, 0.033) are related to Zn homeostasis in the human intestine. The model predicts total daily absorbed Zn (TAZ) (mg Zn d^{-1}) on the basis of total daily dietary PA (TDP) (mmol PA d^{-1}) and total daily dietary Zn (TDZ) (mmol Zn d^{-1}). The TAZ is based on reference adults consuming wheat flour (300 g d^{-1} as either whole grain or its milling fractions) as the sole source of Zn and PA.

2.5. Statistical Analysis

DPS software (DPS 7.05) was used for statistical analysis. The analysis of variance (ANOVA) for the main effects (cropping year, soil N, foliar application) and interactions was determined using the general linear models (GLM) procedure. The means were separated by Fisher's protected least significant difference (LSD) at $p < 0.05$.

3. Results

3.1. Grain Yield and Concentrations of N, PA, P, and K

According to the results of the ANOVA, grain yield and the concentration of N and PA were significantly affected by cropping year and soil N application rate, whereas the different foliar applications significantly influenced N and P concentrations (Table 1, Table 2). Specifically, grain yield was higher in 2015–2016 than in 2014–2015 and greatly increased with the increasing N supply from N0 to N120. Grain N concentration showed a progressive improvement with the increase of N supply from N0 to N240. Grain PA concentration was significantly decreased with the improvement of N supply from N0 to N120 but unaffected by further enhancing N supply from N120 to N240. Additionally, relative to the CK treatment, foliar application of Zn + FA and Zn + AA resulted in 4.1% and 10.3% increase in grain protein concentration, respectively. Grain P concentration was also 32.1% and 21.3% higher in the foliar Zn + SE and Zn + AA treatment, respectively, than the CK treatment.

Table 1. Analysis of variance of experimental factor and their interactions on yield and nutritional quality of winter wheat grain.

Source of Variation	Yield	N	PA	P	K	Total Zn	Soluble Zn
Year (Y)	*	*	**	**	***	*	*
Soil N (N)	***	***	***	NS	NS	**	***
Foliar application (F)	NS	***	NS	*	NS	***	***
Y × N	NS	NS	NS	NS	**	*	NS
Y × F	NS	NS	NS	NS	***	NS	NS
N × F	NS	NS	NS	NS	NS	NS	**

Y × N × F	NS	NS	NS	NS	NS	NS	*
Source of Variation	**Insoluble Zn**	**Fe**	**Mn**	**PA/Zn**	**PA/Fe**	**PA/Mn**	**Estimated Zn Bioavailability**
Year (Y)	**	***	**	**	**	*	***
Soil N (N)	**	**	*	***	***	***	***
Foliar application (F)	***	***	NS	***	***	NS	***
Y × N	**	NS	**	NS	*	NS	**
Y × F	NS	NS	NS	NS	NS	NS	*
N × F	*	NS	NS	***	NS	NS	NS
Y × N × F	NS	NS	NS	NS	NS	NS	NS

NS means not significant. * Significant at $P<0.05$; ** Significant at $P<0.01$; *** Significance at $P<0.001$.

Table 2. Grain yield and N, phytic acid (PA), P, and K concentration of winter wheat, as influenced by soil N fertilizer rate (0 (N0), 120 (N120), or 240 (N240) kg N ha^{-1}) and foliar applications (distilled water spray (CK), 0.4% *w/v* $ZnSO_4 \cdot 7H_2O$ (Zn), 0.4% *w/v* $ZnSO_4 \cdot 7H_2O$ + 0.1% *w/v* fulvic acid (Zn + FA), 0.4% *w/v* $ZnSO_4 \cdot 7H_2O$ + 0.004% *w/v* seaweed extract (Zn + SE), 0.4% *w/v* $ZnSO_4 \cdot 7H_2O$ + 0.01% *w/v* amino acid (Zn + AA), and 0.4% *w/v* $ZnSO_4 \cdot 7H_2O$ + 0.2% *w/v* microbial incubates (Zn + MI)) under field condition in 2014–2015, and 2015–2016.

Treatment	Grain Yield (t ha^{-1})	N (g kg^{-1})	PA (g kg^{-1})	P (g kg^{-1})	K (g kg^{-1})
Year					
2014–2015	4.61 b†	23.5 a	9.03 a	3.63 a	4.25 a
2015–2016	5.03 a	21.9 b	8.17 b	3.06 b	3.93 b
Soil N					
N0	3.73 b	19.3 c	9.85 a	3.28 a	4.12 a
N120	5.39 a	23.3 b	8.10 b	3.30 a	4.08 a
N240	5.35 a	25.6 a	7.85 b	3.44 a	4.06 a
Foliar application					
CK	4.63 a	22.1 c	8.92 a	2.96 c	3.97 c
Zn	4.81 a	22.5 bc	8.52 a	3.29 bc	4.13 abc
Zn + FA	4.92 a	22.9 b	8.34 a	3.33 bc	4.11 abc
Zn + SE	4.86 a	21.9 c	8.55 a	3.91 a	4.01 bc
Zn + AA	4.81 a	24.4 a	8.53 a	3.59 ab	4.11 ab
Zn + MI	4.91 a	22.5 c	8.75 a	2.98 c	4.19 a

†Multiple comparisons among means of two growing years, three soil N rates; and six foliar fertilizer application treatments were tested at $P<0.05$.

3.2. Concentrations of Total Zn and Forms of Zn in Grain

The main effects of year, soil N and foliar applications had significant effects on total, soluble, and insoluble Zn concentration (Table 1). The soil N × foliar applications interaction did not affect total Zn concentration but affected soluble and insoluble Zn concentration (Table 1). Specifically, relative to the CK treatment, foliar application of Zn alone or combined with various biostimulants markedly increased total, soluble, and insoluble Zn concentrations in grain by 64%–45%, 88.6%–162%, and 37%–133%, respectively (Table 3). Compared with the foliar Zn treatment, foliar Zn + FA and Zn + AA treatment resulted in increased total Zn concentration. There was also a positive effect of foliar Zn + AA on soluble Zn concentration for all soil N levels, with the exception of the N120 treatment in 2014–2015 and the N240 in 2015–2016. In both cropping years, the concentration of insoluble Zn in grain was further increased by foliar Zn + AA at N120 rate and further increased by foliar Zn + FA for all soil N levels, with the exception of the N0 treatment in 2014–2015.

Increasing N supply from N0 to N120 caused distinct increase in total Zn concentration in 2015–2016, and further increasing N supply from N120 to N240 resulted in a significant increase in total Zn concentration only in 2014–2015 (Table 3). Soil N application at N120 improved the soluble Zn

concentration relative to the application at N0 in the foliar Zn, Zn + FA (in 2015–2016), and Zn + MI (in 2015–2016) treatments (Table 3). Also, when plants received foliar applications of Zn + FA, Zn + AA, and Zn + MI, the N120 treatment significantly increased grain-insoluble Zn concentration in 2015–2016, but the N240 treatment enhanced insoluble Zn concentration in 2014–2015 in the presence of foliar Zn, Zn + SE, and Zn + MI applications (Table 3). In both years, the highest soluble Zn concentration in grain was for N120 + foliar Zn + AA treatment, while insoluble Zn concentration was highest for N240 + foliar Zn + FA treatment.

Although the soluble Zn concentration in grain was generally lower than insoluble Zn, the increment of soluble Zn concentration following foliar Zn applications relative to the CK treatment was higher than the increment of insoluble Zn (Table 3). In the absence of foliar fertilizer application (CK), the proportion of insoluble Zn in grain was 36%–46%, and foliar fertilizer application increased this proportion to 40%–53% (Figure 1). However, the proportion of insoluble Zn in grain was decreased from 54%–64% to 47%–60% with foliar fertilizer application (Figure 1). In addition, soil N application generally increased the proportion of insoluble Zn (Figure 1).

Table 3. Grain total, soluble, and insoluble Zn concentration of winter wheat as influenced by soil N fertilizer rate (0 (N0), 120 (N120), or 240 (N240) kg N ha^{-1}) and foliar applications (distilled water spray (CK), 0.4% *w/v* $ZnSO_4$ $7H_2O$ (Zn), 0.4% *w/v* $ZnSO_4$ $7H_2O$ + 0.1% *w/v* fulvic acid (Zn + FA), 0.4% *w/v* $ZnSO_4$ $7H_2O$ + 0.004% *w/v* seaweed extract (Zn + SE), 0.4% *w/v* $ZnSO_4$ $7H_2O$ + 0.01% w/v amino acid (Zn + AA), and 0.4% *w/v* $ZnSO_4$ $7H_2O$ + 0.2% *w/v* microbial incubates (Zn + MI)) under field condition in 2014–2015, and 2015–2016.

Foliar	Total Zn (mg kg^{-1})			Soluble Zn (mg kg^{-1})			Insoluble Zn (mg kg^{-1})		
Applications	N0	N120	N240	N0	N120	N240	N0	N120	N240
2014–2015									
CK	20.8 g†	22.0 g	26.7 f	9.5 i	10.0 hi	10.5 h	11.3 i	12.0 i	16.3 h
Zn	42.1 c–e	41.5 e	43.4 b–e	20.1 fg	21.8 abc	21.0 c–f	22.0 d–g	19.6 g	22.4 c–f
Zn + FA	45.5 ab	44.9 abc	46.9 a	20.8 def	21.2 b–e	21.4 a–d	24.6 a–c	23.6 a–d	25.5 a
Zn + SE	40.7 e	41.0 e	43.7 b–e	20.4 efg	20.9 c–f	20.6 d–g	20.2 fg	20.2 fg	23.1 a–e
Zn + AA	43.1 b–e	45.2 ab	45.1 abc	21.1 b-e	22.0 ab	22.3 a	22.1 def	23.2 a–d	22.7 b–e
Zn + MI	43.4 b–e	41.7 de	44.7 a–d	20.6 d–g	21.0 c–f	19.8 g	22.7 b–e	20.7 efg	24.9 ab
Mean	39.3 B	39.4 B	41.8 A	18.7 B	19.5 A	19.3 A	20.5 B	19.9 B	22.5 A
2015–2016									
CK	21.9 f	21.2 f	24.1 f	7.80 h	8.30 gh	8.80 g	14.4 g	12.9 g	15.3 g
Zn	44.3 cde	45.2 cd	45.7 cd	18.9 ef	20.4 bcd	21.4 a	25.4 de	24.8 de	23.4 ef
Zn + FA	45.4 cd	51.5 a	50.1 ab	19.2 ef	21.3 ab	21.2 abc	26.3 cd	30.2 a	28.8 ab
Zn + SE	44.1 de	46.0 cd	47.2 bc	19.5 def	20.3 cd	21.4 a	24.6 de	25.7 cde	25.9 cd
Zn + AA	45.7 cd	52.1 a	46.7 cd	21.5 a	21.8 a	20.2 d	24.2 d–f	30.3 a	26.5 bcd
Zn + MI	41.5 e	46.7 cd	46.6 cd	19.6 de	21.2 abc	18.5 f	21.9 f	25.5 de	28.0 abc
Mean	40.5 B	43.8 A	43.4 A	17.7 B	18.9 A	18.6 A	22.8 B	24.9 A	24.7 A

† Multiple comparisons among the 18 treatments were conducted at $P < 0.05$ (means followed by different lowercase letters). Multiple comparisons among means of three soil N rates were tested at $P < 0.05$ (means followed by different upcase letters).

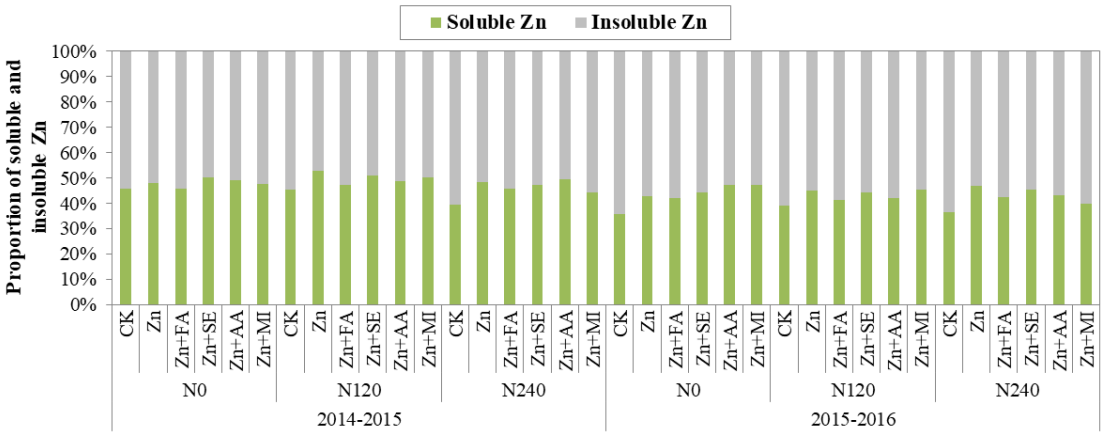

Figure 1. The proportion of soluble and insoluble Zn in winter wheat grain as influenced by soil N fertilizer rate (0(N0),120 (N120), or 240(N240) kg N ha^{-1}) and foliar applications (distilled water spray (CK),0.4% *w/v* $ZnSO_4$ $7H_2O$ (Zn),0.4% *w/v* $ZnSO_4$ $7H_2O$ + 0.1% *w/v* fulvic acid (Zn + FA), 0.4% *w/v* $ZnSO_4$ $7H_2O$ + 0.004% *w/v* seaweed extract (Zn + SE), 0.4% *w/v* $ZnSO_4$ $7H_2O$ + 0.01% *w/v* amino acid (Zn + AA), and 0.4% *w/v* $ZnSO_4$ $7H_2O$ + 0.2% *w/v* microbial incubates (Zn + MI)) under field condition in 2014–2015, and 2015–2016.

3.3. Zn Bioavailability

Compared with the N0 treatment, N120 treatment caused distinct decrease in grain PA/Zn molar ratio at all foliar applications except foliar Zn and Zn + SE (Figure 2). Increasing soil N rate from N120 to N240 further reduced grain PA/Zn molar ratio only when no Zn was sprayed (CK). Irrespective of soil N rates, foliar application of Zn alone or combined with different biostimulants resulted in dramatic decrease in grain PA/Zn molar ratio as compared to the CK (Figure 2). The grain PA/Zn molar ratio did not vary among different foliar treatments at all soil N rates. Inversely, the estimated Zn bioavailability was increased by 106% with foliar fertilizer treatments as compared to the CK treatment (Figure 3). Relative to foliar Zn treatment, foliar Zn + FA treatment further increased estimated Zn bioavailability by an average of 14.6% at N120 and 15.0% at N240. Soil N application at N120 and N240 also resulted in 28.0% and 33.9% increase in grain estimated Zn bioavailability relative to the N0 treatment.

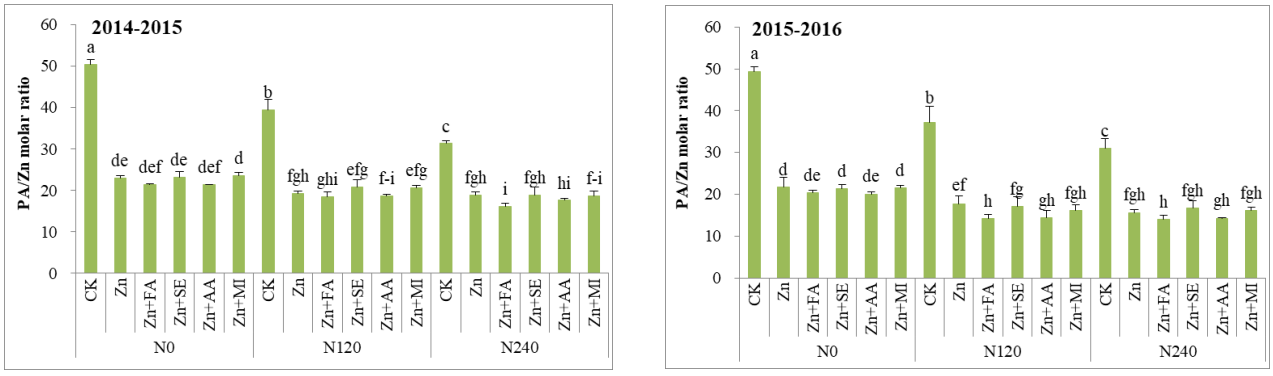

Figure 2. Grain PA/Zn molar ratio of winter wheat as influenced by soil N fertilizer rate (0 (N0), 120 (N120), or 240 (N240) kg N ha^{-1}) and foliar applF[ications (distilled water spray (CK), 0.4% *w/v* $ZnSO_4$ $7H_2O$ (Zn), 0.4% *w/v* $ZnSO_4$ $7H_2O$ + 0.1% *w/v* fulvic acid (Zn + FA), 0.4% *w/v* $ZnSO_4$ $7H_2O$ + 0.004% *w/v* seaweed extract (Zn + SE), 0.4% *w/v* $ZnSO_4$ $7H_2O$ + 0.01% *w/v* amino acid (Zn + AA), and 0.4% *w/v* $ZnSO_4$ $7H_2O$ + 0.2% *w/v* microbial incubates (Zn + MI)) under field condition in 2014–2015, and 2015–2016.

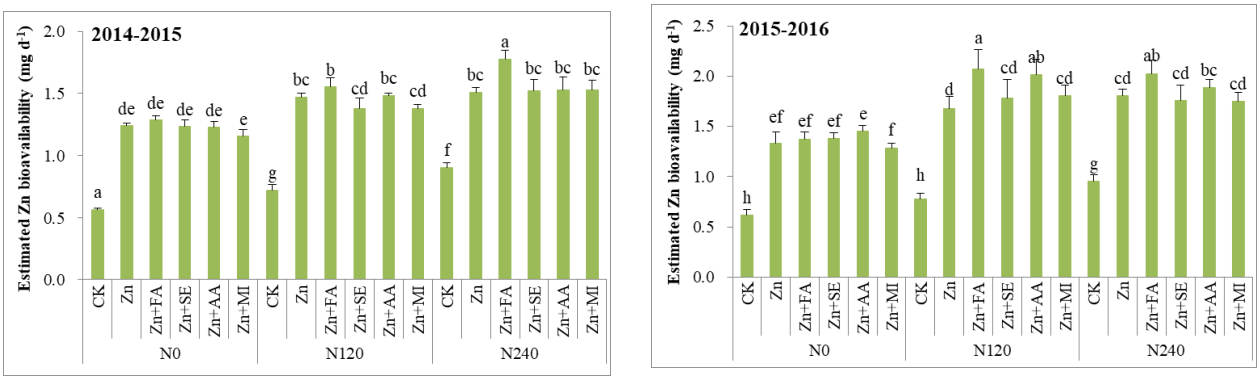

Figure 3. Grain estimated Zn bioavailability of winter wheat as influenced by soil N fertilizer rate (0 (N0), 120 (N120), or 240 (N240) kg N ha^{-1}) and foliar applications (distilled water spray (CK), 0.4% *w*/*v* $ZnSO_4$ $7H_2O$ (Zn), 0.4% *w*/*v* $ZnSO_4$ $7H_2O$ + 0.1% *w*/*v* fulvic acid (Zn + FA), 0.4% *w*/*v* $ZnSO_4$ $7H_2O$ + 0.004% *w*/*v* seaweed extract (Zn + SE), 0.4% *w*/*v* $ZnSO_4$ $7H_2O$ + 0.01% *w*/*v* amino acid (Zn + AA), and 0.4% *w*/*v* $ZnSO_4$ $7H_2O$ + 0.2% *w*/*v* microbial incubates (Zn + MI)) under field condition in 2014–2015, and 2015–2016.

3.4. Fe and Mn Concentration and Bioavailability

The main effects of year, soil N, and foliar applications significantly affected grain Fe concentration and PA/Fe molar ratio (Table 1). Grain Fe concentration increased significantly due to the N120 and N240 treatment in 2014–2015 and due to the N240 treatment in 2015–2016 (Table 4). Grain PA/Fe molar ratio significantly decreased due to the N120 and N240 treatments and was significantly lower in the N240 than in the N120 in 2015–2016. Compared to CK, foliar Zn, Zn + FA, Zn + SE, and Zn + AA treatments significantly increased grain Fe concentration as well as PA/Fe molar ratio during both cropping years.

Grain Mn concentration and PA/Mn ratio were only significantly affected by the main effects of year and soil N (Table 1). Grain Mn concentration in 2014–2015 was higher than that in 2015–2016, while PA/Mn molar ratio was lower in 2014–2015 than in 2015–2016 (Table 4). Compared to the N0 treatment, N120 and N240 did not affect grain Mn concentration in 2014–2015, while significantly decreased grain Mn concentration in 2015–2016 (Table 4). In both years, grain PA/Mn molar ratio was significantly lower in the N120 and N240 treatments than that in the N0 treatment. Foliar Zn treatments did not significantly affect grain Mn concentration and PA/Mn molar ratio in both years (Table 4).

Table 4. Fe, Mn concentration and PA/Fe, PA/Mn molar ratios of winter wheat as influenced by soil N fertilizer rate (0 (N0), 120 (N120), or 240 (N240) kg N ha^{-1}) and foliar applications (distilled water spray (CK), 0.4% *w/v* $ZnSO_4$ $7H_2O$ (Zn), 0.4% *w/v* $ZnSO_4$ $7H_2O$ + 0.1% *w/v* fulvic acid (Zn + FA), 0.4% *w/v* $ZnSO_4$ $7H_2O$ + 0.004% w/v seaweed extract (Zn + SE), 0.4% *w/v* $ZnSO_4$ $7H_2O$ + 0.01% *w/v* amino acid (Zn + AA), and 0.4% *w/v* $ZnSO_4$ $7H_2O$ + 0.2% *w/v* microbial incubates (Zn + MI)) under field condition in 2014–2015, and 2015–2016.

Treatment	Fe (mg kg^{-1})		Mn (mg kg^{-1})		PA/Fe		PA/Mn	
	2014–2015	2015–2016	2014–2015	2015–2016	2014–2015	2015–2016	2014–2015	2015–2016
Soil N								
N0	30.1 c†	38.8 b	36.8 ab	35.3 a	29.1 a	21.0 a	23.2 a	22.6 a
N120	33.6 b	39.2 b	38.8 a	30.4 b	21.8 b	16.6 b	18.7 b	21.2 ab
N240	35.1 a	42.1 a	35.5 b	31.2 b	20.3 b	15.0 c	19.6 b	20.0 b
Foliar application								
CK	30.0 b	37.2 c	37.1 a	31.5 a	26.9 a	19.6 a	21.1 a	22.6 a
Zn	33.2 a	39.8 bc	38.3 a	33.8 a	22.7 bc	17.8 abc	19.2 a	20.4 a
Zn + FA	33.1 a	42.8 a	36.7 a	32.4 a	22.9 bc	15.9 c	20.1 a	20.4 a
Zn + SE	34.8 a	41.2 ab	36.2 a	32.9 a	21.8 c	17.0 bc	20.5 a	20.9 a
Zn + AA	33.7 a	41.2 ab	36.2 a	31.4 a	23.3 bc	16.8 bc	21.1 a	21.3 a
Zn + MI	32.6 ab	38.1 c	37.6 a	31.8 a	24.8 ab	18.2 ab	21.0 a	21.9 a

† Multiple comparisons among means of three soil N rates and six foliar fertilizer application treatments were tested at $P < 0.05$.

4. Discussion

Zinc foliar application alone or together with various biostimulants (FA, SE, AA, MI) significantly enhanced grain Zn concentration and bioavailability. Meanwhile, there were no adverse effects of various biostimulants and Zn on other grain nutritional indices (e.g., N and Mn), suggesting that Zn is compatible with various biostimulants. More importantly, it was observed that adding AA or FA in foliar $ZnSO_4$ application resulted in greater increases in grain Zn as compared to foliar Zn application alone. The average grain Zn concentration obtained by foliar Zn + AA and Zn + FA application was higher than 45 mg kg^{-1}, which highlighted the importance of those two application strategies in achieving adequate Zn concentrations for improved human health. However, in contrast to previous studies [6,11], foliar Zn and biostimulants application in the present study did not affect grain yield. This is possibly because available Zn in the soil is relatively high and plants were not under stressful conditions. Earlier studies have shown that Zn fertilization

may increase the grain yield when available soil Zn was less than 0.40 mg kg^{-1} [27], while our available soil Zn was 0.75 mg kg^{-1}. Similarly, the benefits of diverse biostimulants on plant growth were reported to depend on plant species and environmental conditions, particularly the experience of the plant to stress like salinity, drought, temperature, and oxidative conditions [11]. However, the possible role of various stresses in shaping the yield response to various biostimulants needs to be investigated in the future.

Elevated Zn concentration in grains of wheat sprayed with foliar Zn + FA compared with those sprayed with foliar Zn alone suggests that Zn and FA act synergistically with respect to Zn accumulation when they were both foliar-applied. This synergistic effect might be attributed to the possible contributions of FA to the uptake and/or translocation of foliarly applied Zn in wheat. Consistent with this point, previous studies have shown that FA can complex micronutrients in a soluble form [28] and stimulate their uptake by root or foliage in diverse plants [29,30]. The increment in plant Zn uptake due to FA application has also been reported in cucumber [16] and tomato [31]. In addition, the alteration of plant metabolic processes by the plant growth hormones or plant signaling molecules contained in biostimulants such as FA may also positively influence the movement of Zn in wheat [11]. However, it should be noted that combined foliar application of Zn + FA did not affect soluble Zn concentration in grain. Probably, Zn is transported from vegetative tissues to grain in soluble forms (Zn-nicotianamine (NA) and Zn-deoxymugineic acid (DMA)) but then sequestered in insoluble forms in grain [19]. In line with this, a higher concentration and proportion of water-insoluble Zn in grain was observed with the foliar Zn + FA treatment than with the foliar Zn treatment. These results also indicate that foliar FA application may increase grain total Zn concentration through the increase of Zn sink in the grain. High molecular weight compounds (HMW) such as protein, PA, tannins, and certain types of insoluble fiber are major compounds that may form insoluble complex with Zn in cereal grain [18,26,32]. However, in the present study, enhanced protein or PA concentration cannot explain the observed increase in insoluble Zn concentration in grain flowing foliar Zn + FA application because there were no differences in grain N and PA concentrations between foliar Zn and Zn + FA treatments. Further research is needed to determine compounds that best explain the observed increase in insoluble Zn in grain by foliar Zn + FA application.

In the present study, Zn + AAs produced greater increases both in grain-soluble and -insoluble Zn concentrations in comparison with foliar Zn application alone, resulting in an increase in the total Zn concentration. These results, in combination with the results from Eagling et al. [26] and Li et al. [18], suggest that the large increase in grain Zn concentration caused by foliar Zn + AAs application in the present study may be due to the positive impact of AAs on (i) the formation of soluble Zn complexes (likely Zn-NA/Zn-DMA) in phloem and the improvement of the translocation of Zn from vegetative tissues to grain via the phloem; and (ii) the synthesis of storage compounds such as NA or protein that can interact with Zn in the grain. However, the relative enhancement of grain-soluble and -insoluble Zn concentration in the present study due to foliar Zn + AAs application varied among different soil N rates. Relative to foliar Zn application, foliar Zn + AAs enhanced the grain soluble Zn concentration to a larger extent than the insoluble Zn concentration at N0, while increased insoluble Zn concentration to a larger extent than soluble Zn concentration at N120. Consistent with our finding, Xue et al. [19] concluded that soil N supply provides a larger protein sink for the storage of Zn, and this larger sink may sequester Zn at the expense of the soluble forms. However, in the absence of soil N supply, it is possible that the application of AAs improved nitrogen compounds (NA/DMA) or proteins that could form soluble complexes with Zn, which thereby resulted in larger increase in soluble Zn concentrations in grain.

The results of the present study, in which the addition of SE or MI did not enhance the effects of the foliar Zn applications on grain concentration, are in part contradicting with the previous reports. For example, Tian et al. [14] reported that foliar application of microbial incubates greatly enhanced the uptake and movement of foliar-applied Zn in sunflower. Several studies on horticultural crops have also shown that soil applications of seaweed extracts were able to improve uptake of Zn and other nutrients [33–35]. The most probable reason for these variable outcomes may be the different

plant species and application methods. As concluded by Kunicki et al. [36], the effect of a biostimulant can differ among plant types and depends on environmental factors and the time of application. In addition, the diverse sources of biostimulants may be the reason affecting the efficiency of biostimulants. It is known that the molecules contained in the biostimulants derived from various materials can be different [37]. However, due to the complexity of the biostimulants, their specific functional molecules are poorly determined [38], so more work is still needed in this regard.

The bioavailability of Zn in food is as important as its total concentration. The PA/Zn molar ratio in grain, mostly reflecting Zn concentration, was decreased significantly by foliar application of Zn alone or together with biostimulants, confirming earlier results [24] and indicating enhanced Zn bioavailability. According to dietary Zn intake standards for adult humans, 3 mg Zn/300 g grain is the target level for Zn bio-fortification [39]. In the present study, foliar applications of Zn combined with FA or AAs are the most efficient approaches to increase the estimated grain Zn bioavailability. However, 300 g of grain could provide only about 56% and 50% of the daily Zn requirement with application of foliar Zn + FA or Zn + AAs, respectively. Furthermore, a combination of high soil N application (N240) with foliar applications (Zn + FA or Zn + AAs) resulted in the highest estimated Zn bioavailability in grain. Besides, with high soil N application, grain concentrations of Fe and N increased, whereas antinutrient PA concentration decreased, which could further contribute to human health. However, it is noteworthy that the local farmer's commonly used soil N rates (240 kg ha^{-1}) were excessive, and no yield benefit was observed beyond the reduced soil N rate (120 kg ha^{-1}). Additionally, the overuse of N fertilizer in intensive agricultural areas of China has led to a series of environmental problems, including eutrophication, greenhouse gas emissions, soil acidification, and groundwater pollution [20,40]. Thus, the reduced soil N rate (120 kg ha^{-1}) should be used because it is more economically viable and environmentally friendly. Wang et al. [10] reported that adding urea in foliar Zn application can be an efficient approach to further increase grain Zn concentration and bioavailability under reduced soil N levels. Thus, further studies are needed to evaluate the combined effects of foliar Zn, biostimulant, and urea under reduced soil N rates. Moreover, it is important to highlight that the concentration and bioavailability of Zn may also be affected by the level of other antinutritional compounds (such as fiber and tannins) and the processing (milling, fermentation, heating, etc.) of wheat grain to final products. For example, Cubadda et al. [41] reported that milling of whole grain (durum wheat) to flour can lead to 66% loss of Zn. Bilgiçli et al. [42] found that more than 94% of PA was degraded in the processing of flour to tarhana. Thus, further studies are still required in this regard to comprehensively evaluate of the gains in nutritional quality of grains from Zn biofortification.

5. Conclusions

Foliar $ZnSO_4$ application alone or combined with fulvic acid (FA), seaweed extracts (SE), amino acid (AA), and microbial incubates (MI) significantly enhanced grain Zn concentration and bioavailability in both years. Compared to foliar Zn alone, foliar Zn + FA and Zn + AAs application caused higher increase in grain Zn than foliar Zn alone. The higher increase of grain Zn following foliar Zn+FA than foliar Zn only was mainly from insoluble Zn increases, while the corresponding Zn increase following foliar Zn + AAs was both from soluble (at N0) and insoluble Zn (at N120). Foliar application of Zn + FA and Zn + AAs generally resulted in higher Zn bioavailability than foliar Zn alone. Moreover, grain N concentration and Fe concentration and bioavailability were significantly improved due to foliar Zn + AAs and soil N supply. The grain nutritional indices including Mn, N, PA, P, and K were higher in 2014–2015 than in 2015–2016. In general, foliar Zn plus FA or AAs (with appropriate soil N management) may be a promising strategy for improving total and bioavailable Zn concentrations of wheat grain also having important co-benefits.

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