

Article

# Enhancing Zinc Accumulation and Bioavailability in Wheat Grains by Integrated Zinc and Pesticide Application

Peng Ning <sup>1</sup>, Shaoxia Wang <sup>2</sup>, Peiwen Fei <sup>1</sup>, Xiaoyuan Zhang <sup>1</sup>, Jinjin Dong <sup>1</sup>, Jianglan Shi <sup>1</sup> and Xiaohong Tian <sup>1,\*</sup>

<sup>1</sup> College of Natural Resources and Environment, Northwest A&F University, Yangling 712100, Shaanxi, China

<sup>2</sup> College of Resources and Environmental Science, Qingdao Agricultural University, Qingdao 266109, Shandong, China

\* Correspondence: txhong@nwafu.edu.cn; Tel.: +86-29-8708-2948

Received: 1 August 2019; Accepted: 9 September 2019; Published: 11 September 2019



**Abstract:** Incorporating foliar zinc (Zn) spray into existing pesticide application is considered highly cost-effective to biofortify wheat (*Triticum aestivum*) with Zn. However, the effectiveness of this combined approach in terms of Zn enrichment and bioavailability in grain and its milling fractions is not well examined. Two-year field experiments were conducted in 2017 and 2018 with three sets of foliar applications (nil Zn as control, foliar Zn alone, and foliar Zn plus pesticides) at the anthesis, milk stage, or both. Compared to the control, grain yield was not affected by foliar Zn application alone or combined with pesticides, while the Zn concentrations and bioavailability substantially increased in the whole-grain, bran, and flour irrespective of spray timing. Yield losses by 28%–39% (2018 vs. 2017) led to 7%–18% and 18%–38% increase of Zn density in grain and flour, respectively. Further, such negative responses were uncoupled by foliar spray of Zn or Zn plus pesticides, and absent from the control plants. Nonetheless, grain Zn biofortification was achieved in both low- and high-yield plants with either Zn spray alone or combined with pesticides. Together with the enhanced Zn bioavailability in grain, bran, and flour, the effectiveness of this combined strategy is validated to biofortify wheat with Zn.

**Keywords:** agronomic biofortification; foliar zinc application; pesticide; zinc bioavailability; wheat

## 1. Introduction

It is estimated that about two billion of the world's population are zinc (Zn) deficient, especially in the developing countries [1–3]. Chronic inadequate Zn intake particularly from cereal-based diets is the major cause of Zn malnutrition, and biofortification is considered an effective strategy to overcome this issue through increasing Zn concentrations in edible crops [4,5]. Wheat (*Triticum aestivum* L.), representing an important staple food crop worldwide, has inherently low Zn concentrations in grain [6–8]. Currently, grain Zn in wheat grown in major producing regions ranges from 20 to 30 mg kg<sup>-1</sup> with an average of 27.3 mg kg<sup>-1</sup> at global scale, leaving a wide gap to the biofortification target for human health (40–60 mg kg<sup>-1</sup>) [3,5,9]. The Zn bioavailability in wheat grains is also relatively low due to the presence of antinutritional compounds such as phytic acid [10]. Therefore, it is crucial to biofortify wheat with Zn to avoid this micronutrient deficiency risk for human health [1,5].

Two major strategies to biofortify food crops with Zn are agronomic interventions including appropriate fertilizer application and genetic improvement such as plant breeding and genetic modification [3,4]. It has been well documented that foliar spray of Zn fertilizer (commonly zinc sulfate) is a preferable agronomic option and outperforms the direct application to soils or seed

priming [4,11,12]. However, if Zn is not a growth-limiting factor in soils, farmers would not be motivated to spray Zn due to extra labor costs and the lack of obvious yield benefits [13]. Under this circumstance, highly cost-effective options have been established by incorporating foliar Zn spray into existing pesticide application [14–17]. It has been validated in wheat, rice, and common beans in various locations, and been concluded that Zn and pesticides can be compatible without any adverse effects on grain Zn enrichment or pest control [15–17].

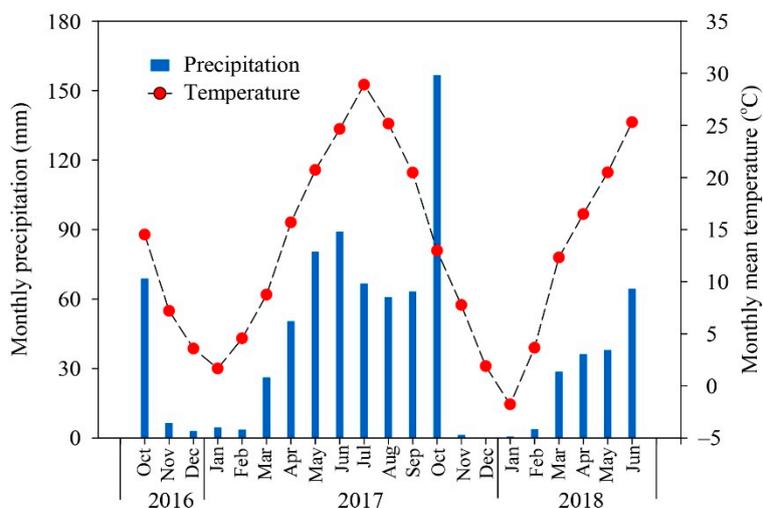
Nonetheless, the magnitude of the increase in grain Zn through either foliar Zn spray alone or in combination with pesticides may vary among diverse environments, crop species, and even in a specific condition [13,16]. A multi-site/year field study has found that foliar application of Zn plus pesticides significantly reduced effectiveness of foliar Zn application in increasing grain Zn concentration in six out of 24 field sites [16]. Further, the timing of foliar Zn spray is an important factor influencing its effectiveness of biofortification, which is particularly effective at a later rather than an earlier developmental stage, preferably during grain filling [4,18]. However, it remains unclear whether the spray timing affects the effectiveness of foliar application of Zn plus pesticides. In addition, very few studies have investigated the impacts of Zn plus pesticide application on the Zn enrichment and bioavailability in the mainly consumed grain fraction (flour) so far [17].

To address these questions, we conducted two-year field experiments to examine the effectiveness of foliar applications of Zn in combination with various commonly used insecticide/fungicide, in terms of Zn concentration and bioavailability in both grain and its fractions. Besides, influences of foliar spray timing were also explored at anthesis, milk stage, or both. Considering the widespread global health burden by Zn deficiency, such a cost-effective approach is practical for farmers to biofortify wheat with Zn, as well as other cereal crops, to combat this malnutrition issue.

## 2. Materials and Methods

### 2.1. Site Description

Field experiments were conducted from October 2016 to June 2018 at the Doukou Experimental Station of Northwest A and F University (34°36' N, 108°52' E), Shaanxi Province, China. The soil type at the study site was an Earth-cumuli Orthic Anthrosol. Pre-plant soil chemical properties in the top 20 cm layer were as follows: pH 8.4, organic matter 18.3 g kg<sup>-1</sup>, total N 1.42 g kg<sup>-1</sup>, Olsen P 32.0 mg kg<sup>-1</sup>, ammonium acetate-extractable K 183.0 mg kg<sup>-1</sup>, and diethylene triamine pentaacetic acid extractable Zn (DTPA-Zn) 0.91 mg kg<sup>-1</sup>. The monthly precipitation and mean temperature during the growing seasons are presented in Figure 1.



**Figure 1.** The monthly precipitation and mean temperature during wheat growing seasons in 2016–2018.

## 2.2. Experimental Design

Winter wheat (*Triticum aestivum* L., cv Huaimai 22) was planted in mid-October of 2016 and 2017, and harvested in the early June of 2017 and 2018, respectively. The seeding rate was 210–225 kg ha<sup>-1</sup> and inter-row spacing was 15 cm. Nine treatments considering three sets of foliar applications (nil Zn as control, foliar Zn alone, and foliar Zn + pesticides) at three spray timing (at anthesis, milk, or both) were performed in the present study, and were imposed in a randomized complete block design with four replications. Three foliar Zn application treatments included spray of 0.4% (w/v) ZnSO<sub>4</sub>·7H<sub>2</sub>O solution twice at the wheat anthesis stage (Zn<sub>A</sub>), twice at the milk stage (Zn<sub>M</sub>), and split as single spray at anthesis and milk stage (Zn<sub>A+M</sub>). Three Zn plus pesticide mixture treatments consisted of twice sprayed Zn plus fungicide thiophanate-methyl and insecticide beta-cypermethrin at anthesis (P<sub>1</sub>Zn<sub>A</sub>), twice sprayed Zn plus fungicide triadimefon and insecticide imidacloprid at milk stage (P<sub>2</sub>Zn<sub>M</sub>), and split as a single spray at each stage (P<sub>12</sub>Zn<sub>A+M</sub>). Three corresponding control treatments with foliar spray of 0.01% (v/v) Tween 20, instead of Zn and pesticides application were conducted at anthesis (Ctrl<sub>A</sub>), at milk stage (Ctrl<sub>M</sub>), and at both anthesis and milk stage (Ctrl<sub>A+M</sub>).

The application rate of 0.4% (w/v) ZnSO<sub>4</sub>·7H<sub>2</sub>O was designed according to the previous field trials conducted at diverse locations of China [15,17], which was effective to biofortify wheat with Zn and without Zn toxicity. The pesticide concentration was 0.2% (v/v) for thiophanate-methyl, 0.07% (v/v) for beta-cypermethrin, 0.2% (v/v) for triadimefon, and 0.1% (v/v) for imidacloprid according to the manufacturer's instructions, respectively. Previous study has shown that Zn concentration is not affected when mixed with these pesticides [17]. All foliar solutions contained 0.01% (v/v) Tween 20 as surfactant applied at a rate of 1250 L ha<sup>-1</sup>. Foliar application was performed in 2 m × 1 m micro-plots at the end of day, and the same quantity of Zn was applied for foliar Zn treatments with or without pesticides. Additionally, 127 kg N ha<sup>-1</sup> as urea and 100 kg P<sub>2</sub>O<sub>5</sub> ha<sup>-1</sup> as superphosphate were applied at planting for all treatments each year.

## 2.3. Plant Sampling and Analyses

At maturity of wheat, grains were separated from straw manually and yield was estimated based on the entire micro-plot each year. Subsamples of grains were washed thoroughly with tap water followed by deionized water, and dried at 65 °C to a constant weight. Approximately 150 g grain were collected and used for milling. Grain moisture was adjusted to 15% with distilled water and kept for 36 h before milling. Grains were fractionated into bran and flour using a Quadrumat Junior mill (Brabender, Duisburg, Germany). In addition, another 20 g whole-grain subsamples were ground using a ball mill for further analysis.

The ground grain, flour, and bran samples were combusted at 550 °C for 6 h, and then dissolved in 1:1 (v:v) HNO<sub>3</sub> solution for Zn assay using atomic absorption spectrometry (AA320CRT, Kexiao Scientific Instruments Co. LTD, Shanghai, China). Phytic acid concentrations of these samples were determined as previously described [17], which was based on precipitation of ferric phytate and measurement of iron (Fe) remaining in the supernatant. Further, N concentrations of whole grain and its fractions were determined using a modified Kjeldahl digestion method [19], and converted to protein concentrations by a coefficient of 5.7.

## 2.4. Estimation of Zn Bioavailability

The tri-variate model of Zn absorption considering total daily-absorbed Zn (TAZ) by a human was used to estimate Zn bioavailability in whole grain and flour as previously described [17,20].

$$\text{TAZ} = 0.5 \times 65 \times 100 \times \left( A_{\text{MAX}} + \text{TDZ} + K_{\text{R}} \times \left( 1 + \frac{\text{TDP}}{K_{\text{P}}} \right) - \sqrt{\left( A_{\text{MAX}} + \text{TDZ} + K_{\text{R}} \times \left( 1 + \frac{\text{TDP}}{K_{\text{P}}} \right) \right)^2 - 4 \times A_{\text{MAX}} \times \text{TDZ}} \right) \quad (1)$$

The model predicts TAZ (mg Zn d<sup>-1</sup>, termed “estimated Zn bioavailability”) on the basis of total daily dietary phytate (PA) (TDP, mmol PA d<sup>-1</sup>) and total daily dietary Zn (TDZ, mmol Zn d<sup>-1</sup>),

which assumes a reference adult consuming 300 g wheat flour per day as the sole source of Zn and PA. The parameters maximum absorption ( $A_{MAX}$ , 0.091), equilibrium dissociation constant of Zn-receptor binding reaction ( $K_R$ , 0.680), and equilibrium dissociation constant of Zn-PA binding reaction ( $K_P$ , 0.033) are related to Zn homeostasis in human intestine.

### 2.5. Statistical Analyses

For each variable, the effects of foliar spray treatments and year were analyzed with two-way ANOVA in SAS (SAS Institute, Cary, NC, USA). The treatment and year factors were considered as fixed, and replication as random factors. Treatment means were compared based on least significant difference (LSD) at the 0.05 level of probability. The correlation analysis of grain Zn uptake and concentration against grain yield was performed in SigmaPlot for Windows Version 12.0 (Systat Software, Inc.).

## 3. Results

### 3.1. Grain Yield and Grain Zn Uptake and Concentration

The significance level for all measured variables is shown in Table 1. Variation due to year was observed for parameters except bran Zn concentration and flour Zn bioavailability. Zinc uptake, concentrations, and bioavailability in grain and its fractions exhibited significant changes due to Zn treatment, while no year  $\times$  treatment interactions were found for all parameters (Table 1).

**Table 1.** Significance level of the fixed effects for each of the measured variables.

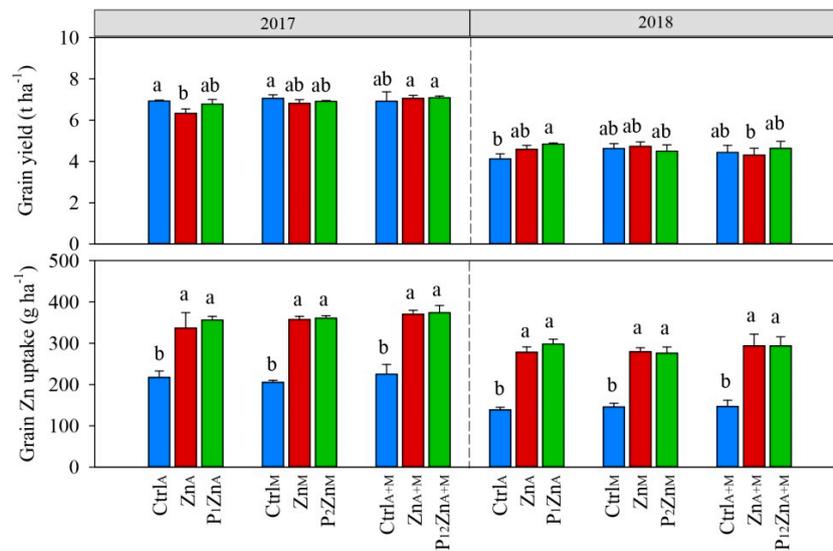
Measured Variable	Source of Variation		
	Year (Yr)	Treatment (T)	Yr $\times$ T
Grain yield (t ha <sup>-1</sup> )	<0.0001	n.s.	n.s.
Grain Zn uptake (g ha <sup>-1</sup> )	<0.0001	<0.0001	n.s.
Grain Zn concentration (mg kg <sup>-1</sup> )	<0.0001	<0.0001	n.s.
Bran Zn concentration (mg kg <sup>-1</sup> )	n.s. <sup>1</sup>	<0.0001	n.s.
Flour Zn concentration (mg kg <sup>-1</sup> )	<0.0001	<0.0001	n.s.
Grain protein concentration (g kg <sup>-1</sup> )	<0.0001	n.s.	n.s.
Bran protein concentration (g kg <sup>-1</sup> )	<0.0001	n.s.	n.s.
Flour protein concentration (g kg <sup>-1</sup> )	<0.0001	n.s.	n.s.
Grain phytate concentration (g kg <sup>-1</sup> )	<0.0001	n.s.	n.s.
Bran phytate concentration (g kg <sup>-1</sup> )	<0.0001	n.s.	n.s.
Flour phytate concentration (g kg <sup>-1</sup> )	<0.0001	n.s.	n.s.
Zn bioavailability in grain (mg Zn d <sup>-1</sup> )	0.0041	<0.0001	n.s.
Zn bioavailability in bran (mg Zn d <sup>-1</sup> )	0.0005	<0.0001	n.s.
Zn bioavailability in flour (mg Zn d <sup>-1</sup> )	n.s.	<0.0001	n.s.

<sup>1</sup> n.s., not significant ( $p > 0.05$ ).

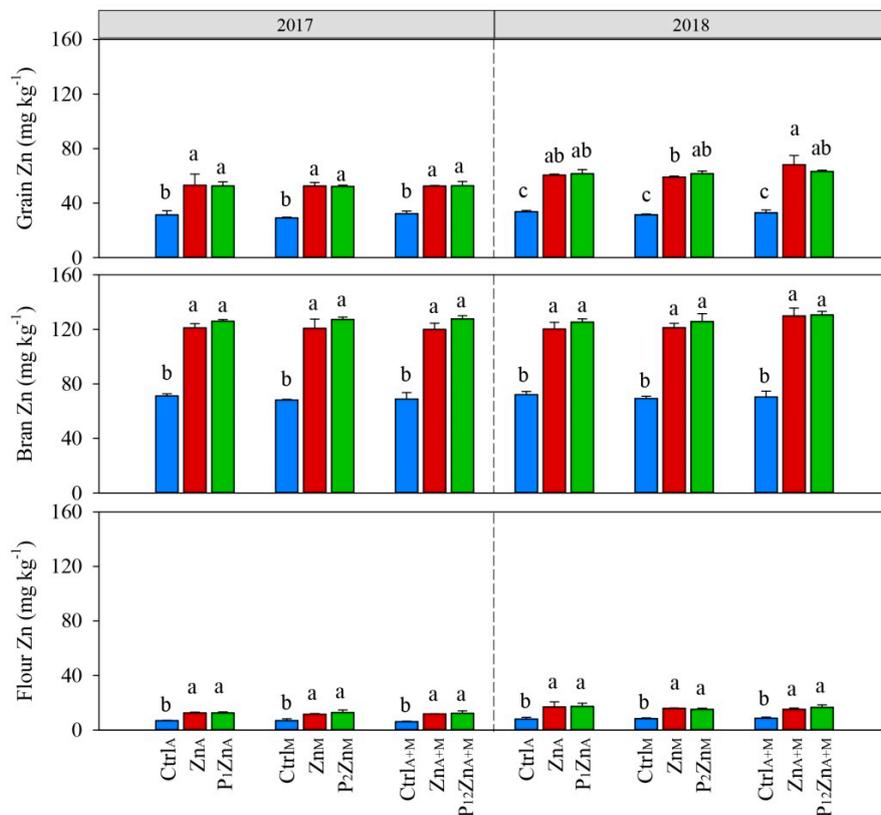
Wheat grain yield ranged from 6.3 to 7.1 t ha<sup>-1</sup> in 2017, which was 38%–69% greater than in 2018. Foliar Zn spray alone or with pesticides had very limited impacts on grain yield in both years with a few exceptions (Figure 2). In contrast, compared to the control groups (Ctrl<sub>A</sub>, Ctrl<sub>M</sub> or Ctrl<sub>A+M</sub>), grain Zn uptake in treatments with foliar Zn application at the anthesis (Zn<sub>A</sub>), milk (Zn<sub>M</sub>), or split at each stage (Zn<sub>A+M</sub>) increased by 0.5- to 0.8-fold in 2017 and by 0.9- to 1.1-fold in 2018, respectively (Figure 2). Similar responses were observed in treatments with Zn plus pesticide spray, i.e., P<sub>1</sub>Zn<sub>A</sub>, P<sub>2</sub>Zn<sub>M</sub>, and P<sub>12</sub>Zn<sub>A+M</sub>. Grain Zn uptake in 2017 was 41%–59% more in control groups than that in 2018, and 20%–31% more in Zn or Zn plus pesticides groups, respectively.

Grain Zn concentration in the control groups varied between 29.1 to 33.7 mg kg<sup>-1</sup> in the two years, which was improved to 52.5 to 68.2 mg kg<sup>-1</sup> by foliar Zn application with or without pesticides. Similar patterns were found for Zn concentrations in bran and in flour (Figure 3). Across years and treatments, foliar Zn application alone or in combination with pesticides resulted in an 81% increase of Zn concentration in grain than the control on average, as well as 78% increase in bran, and 89% increase in flour, respectively. Overall, Zn concentration in bran was not varied with years, but increased by

2%–30% in grain and by 18%–41% in flour in 2018 than in 2017 (Figure 3). There were very limited variations among spray timing within groups of control, Zn alone, or Zn plus pesticides.



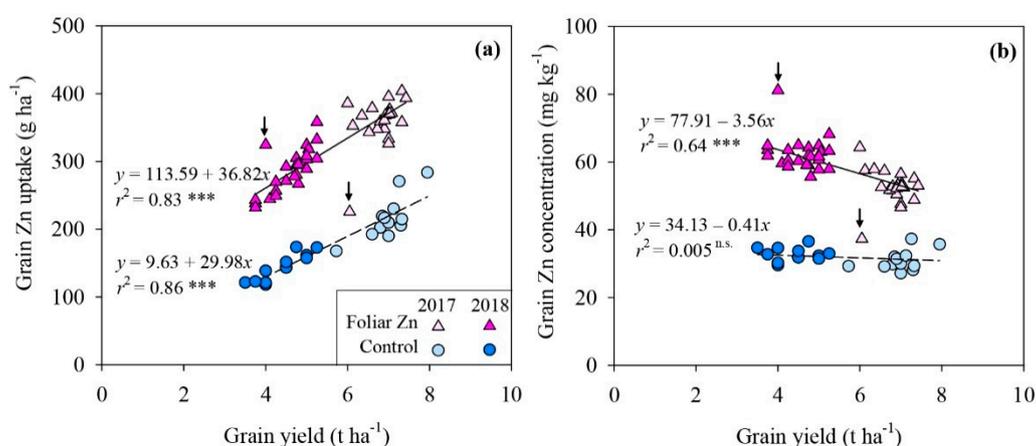
**Figure 2.** The grain yield and grain Zn uptake in wheat plants harvested in 2017 and 2018. Treatments consisted of nil Zn spray (control, Ctrl) and foliar Zn application alone (Zn) or plus pesticides (PZn) at the anthesis (A), milk stage (M) or both (A+M). Pesticides were a mixture of fungicide + insecticide which were thiophanate-methyl + beta-cypermethrin at anthesis (P<sub>1</sub>Zn<sub>A</sub>) and triadimefon + imidacloprid at milk stage (P<sub>2</sub>Zn<sub>M</sub>). Vertical bars represent the standard error of four replications. Columns with no letter in common indicate significant differences between treatments each year by least significant difference (LSD) test ( $p < 0.05$ ).



**Figure 3.** Zinc concentrations in grain, bran, and flour of wheat plants harvested in 2017 and 2018. Treatments consist of nil Zn spray (control, Ctrl) and foliar Zn application alone (Zn) or plus pesticides

(PZn) at the anthesis (A), milk stage (M) or both (A+M). Pesticides were a mixture of fungicide + insecticide which were thiophanate-methyl + beta-cypermethrin at anthesis (P<sub>1</sub>Zn<sub>A</sub>) and triadimefon + imidacloprid at milk stage (P<sub>2</sub>Zn<sub>M</sub>). Vertical bars represent the standard error of four replications. Columns with no letter in common indicate significant differences between treatments each year by LSD test ( $p < 0.05$ ).

Across the two-year data, strong and positive correlations were found between grain yield and grain Zn uptake in both control groups and foliar Zn spray groups ( $r^2 = 0.83$  to  $0.86$ ,  $p < 0.001$ ) (Figure 4a). However, the correlation between grain Zn concentration and yield was negative and only significant in the foliar Zn treatments ( $r^2 = 0.64$ ,  $p < 0.001$ ), and not in the plants without Zn application ( $p > 0.05$ ) (Figure 4b).

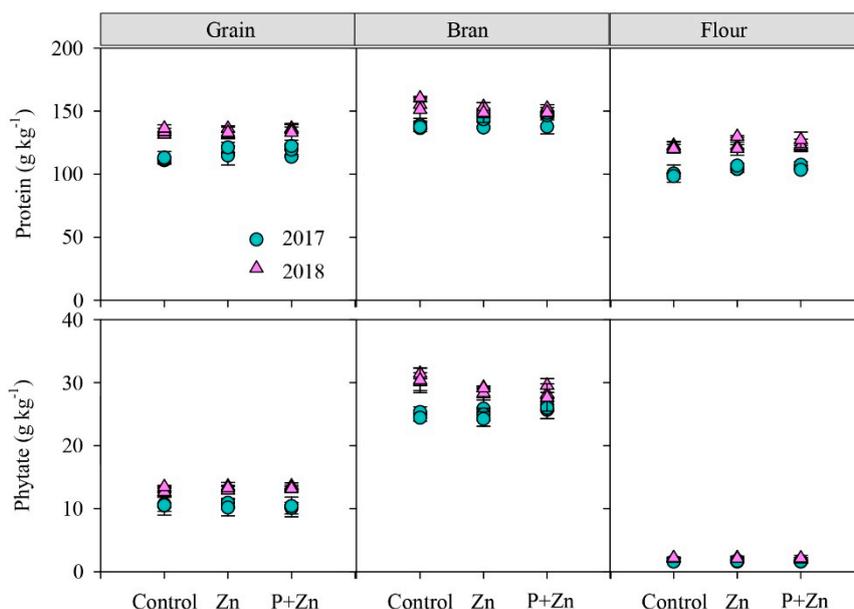


**Figure 4.** The correlation analysis of grain Zn uptake (a) and concentration (b) against grain yield in wheat with nil Zn application (control) and foliar Zn spray (with and without pesticides). The arrow pointing data were removed from the trend-line to give a higher  $r^2$ . \*\*\*,  $p < 0.001$ ; n.s., not significant.

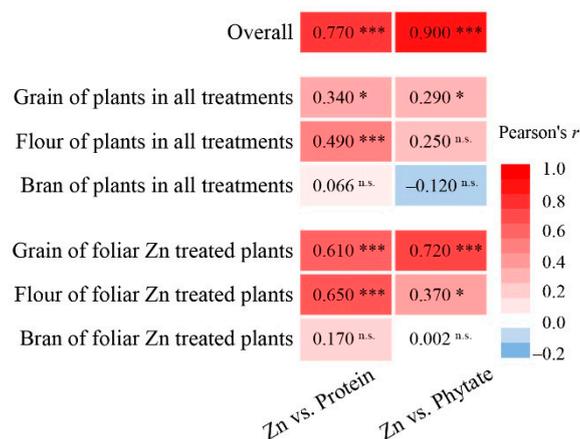
### 3.2. Zn-Binding Ligands in Grain and Its Fractions

Regarding the Zn-binding ligands in grain and its fractions, neither the protein nor phytate concentrations differed between Zn spray treatments (Figure 5). However, higher grain yield in 2017 led to lower protein and phytate concentrations in grain, bran, and flour, when compared to those in 2018. In addition, foliar Zn (or Zn plus pesticides) application tended to increase flour protein concentrations (Figure 5).

Across all years and treatments, whole-grain Zn concentration was positively correlated with protein concentration ( $r^2 = 0.340$ ,  $p < 0.05$ ) and phytate concentration ( $r^2 = 0.290$ ,  $p < 0.05$ ) (Figure 6). Further, such correlation was stronger in grains of foliar Zn spray treated plants (with and without pesticides), i.e.,  $r^2 = 0.610$  ( $p < 0.001$ ) for protein and  $r^2 = 0.720$  ( $p < 0.001$ ) for phytate. Similar responses were exhibited in the flour fraction. However, Zn concentrations in bran were not associated with protein or phytate concentrations in all treatments, as well as in the foliar Zn spray treated plants (Figure 6).



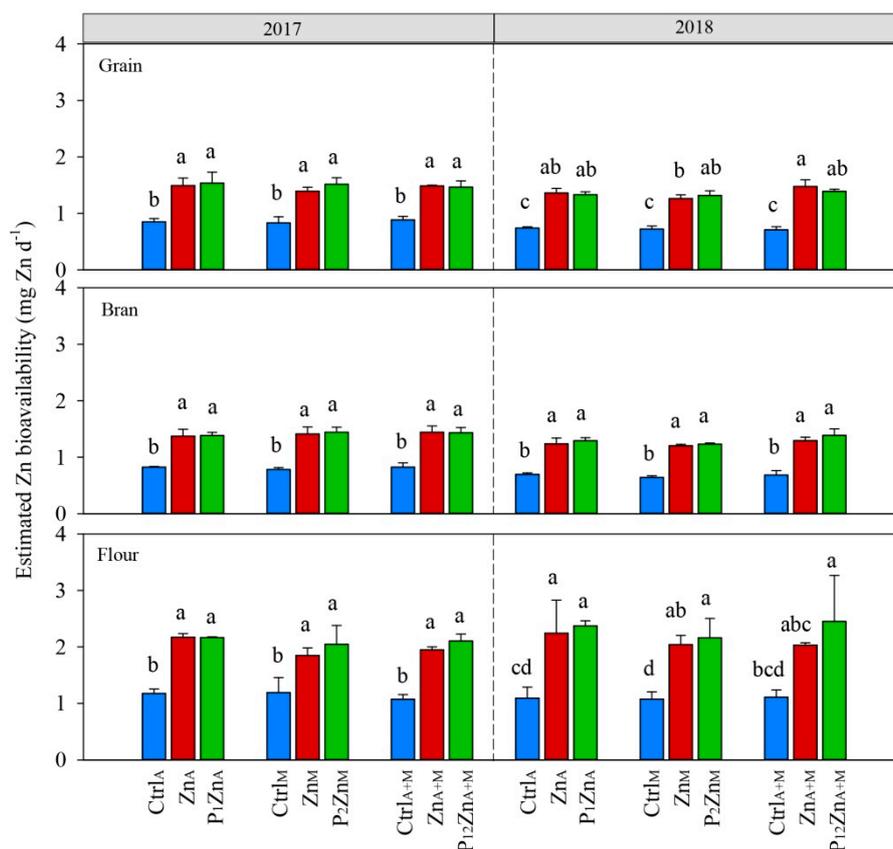
**Figure 5.** The protein and phytate concentrations in grain, bran, and flour in wheat with nil Zn spray (control) and foliar Zn application alone (Zn) or plus pesticides (P+Zn) in 2017 and 2018. Vertical bars represent the standard error of four replications.



**Figure 6.** Pearson correlation coefficients between Zn and protein concentrations and between Zn and phytate concentrations in grain, bran, and flour of wheat plants from all treatments (control, foliar Zn, and Zn plus pesticides) or from foliar Zn spray treated plants (with and without pesticides). \*,  $p < 0.05$ ; \*\*\*,  $p < 0.001$ ; n.s., not significant.

### 3.3. Zinc Bioavailability

Zinc bioavailability was estimated using the tri-variate model [20]. Compared to the control groups, grain Zn bioavailability in plants with foliar Zn or Zn plus pesticide applications either at anthesis or milk stage was significantly increased by 65%–82% in 2017 and by 75%–108% in 2018, respectively (Figure 7). There were limited variations for Zn bioavailability between foliar Zn and Zn plus pesticide applications, and between Zn spray timing. Similar patterns were found in both bran and flour fractions. The estimated Zn bioavailability in flour was about 1.1 mg Zn d<sup>-1</sup> in the control groups on average, which was nearly doubled by foliar Zn application with or without pesticides (Figure 7).



**Figure 7.** The estimated Zn bioavailability in whole-grain and its milling fractions (bran and flour) of wheat harvested in 2017 and 2018. Treatments consisted of nil Zn spray (control, Ctrl) and foliar Zn application alone (Zn) or plus pesticides (PZn) at the anthesis (A), milk stage (M) or both (A+M). Pesticides were a mixture of fungicide + insecticide which were thiophanate-methyl + beta-cypermethrin at anthesis (P<sub>1</sub>ZnA) and triadimefon + imidacloprid at milk stage (P<sub>2</sub>ZnM). Vertical bars represent the standard error of four replications. Columns with no letter in common indicate significant differences between treatments each year by LSD test ( $p < 0.05$ ).

#### 4. Discussion

Agronomic biofortification with Zn, particularly through foliar applications, has been proved very effective for wheat and also other cereal crops [4,21–23]. It provides a practical approach to combat the global Zn malnutrition in populations with cereal-based diets [2,4,24]. Although the present soil was not Zn deficient (DTPA–Zn 0.91 mg kg<sup>-1</sup>) and grain yield was not affected by Zn applications, grain Zn in wheat plants without foliar Zn spray ranged from 29–34 mg kg<sup>-1</sup> across years (Figure 3). It is comparable to the global scale of 20 to 35 mg kg<sup>-1</sup> [6,8,13,25,26], showing a solid gap to the biofortification target for human health (40–60 mg kg<sup>-1</sup>) [3,5,9]. By foliar Zn application, grain Zn concentration increased by 62%–81% in 2017 and by 80%–107% in 2018 than the control plants, respectively (Figure 3). Such effectiveness far over 10 mg kg<sup>-1</sup> by foliar Zn spray suggests a measurable biological impact on human Zn nutrition [5,9,18]. In agreement with other reports, the increases in grain Zn by foliar Zn spray occurs also in grain milling fractions such as bran and flour with a similar magnitude (Figure 3) [11,17,27–29]. Certainly, foliar spray of Zn fertilizers represents a fast and effective strategy to biofortify wheat with Zn.

However, if there is no yield-limiting Zn deficiency problem in soils and no premium price of Zn-biofortified grains, farmers would not be motivated to spray Zn due to extra labor costs and investments [13]. In such a situation, a few studies have developed highly cost-effective agronomic solutions of Zn biofortification, e.g., the combined foliar application of Zn fertilizer plus pesticides [14–17]. Spray of insecticide/fungicide is a widely accepted management of pest control in

wheat production in China, to either prevent or cure diseases and insect pests. Pesticides are commonly applied at the anthesis and early milk stages when the peak infestation occurs [15], which is well matched with the best application times to maximize the grain Zn accumulation [3,18]. The present study corroborates that foliar application of pesticides together with Zn fertilizer is a worthwhile and practical strategy to improve Zn level in grain and in its milling fractions, since similar effectiveness was obtained by foliar Zn application with or without pesticides (Figures 2 and 3) [14–16]. In turn, no observed differences in grain yield between these two managements indicated that the common pesticides can be mixed with Zn fertilizer without any antagonisms, which is supported by the absence of any adverse interactions between Zn and insecticides regarding the toxic effect of insecticides [15]. In addition, it might be associated with the fact no obvious pests and diseases occurred in both years [17].

Further, in both wheat and rice, foliar Zn applications are particularly effective in enriching the grain with Zn if they are applied at a later rather than an earlier developmental stage, preferably during grain-filling [4,18]. A recent study has also found that foliar Zn application (with or without pesticides) at milk stage can result in a higher Zn concentration and bioavailability in whole grain than Zn applied at flowering stage in wheat [17]. However, this was not the case in the present study where spray timing (twice at anthesis, twice at milk stage, or split at anthesis and milk stages) had no influences on the Zn concentrations in whole-grain, or bran and flour fractions (Figure 3). The results suggested that foliar Zn sprays at either anthesis or milk stages are both applicable and effective to biofortify wheat with Zn [16,26]. Likewise, relative to the control groups, foliar Zn application either alone or together with different pesticides led to 0.53- to 1.21-fold greater Zn bioavailability in grain and its milling fractions, and such responses were much stable among Zn spray timing (Figure 7). Taken together, the present results, as well as other field studies with wheat and rice conducted in diverse environments, have clearly demonstrated that foliar Zn application or in combination with pesticides are highly effective solutions to increase grain Zn levels [12–14,16,24].

Grain yield and Zn concentration in wheat often varies greatly depending on management, genotypes, and soil and other environmental conditions [6,8,13,16,26]. It has been well documented that large increases in yield have caused considerable reduction in concentrations of essential nutrients including Zn [6–8]. Similarly, corresponding to the lower grain yield in 2018 than in 2017 in the present study, Zn concentration was increased by 7%–18% in the whole-grain and by 18%–38% in the flour, respectively (Figure 3). A further correlation analysis revealed that such responses of grain Zn concentrations to yield were uncoupled by Zn availability during grain filling, and only responsive when adequate Zn was sprayed but not in the control groups (Figure 4). These results implied that, under natural or field conditions, grain Zn is usually source-limited and mainly affected by the Zn reserves in vegetative tissues and its remobilization to grain [4,13,30]. Thus, as shown in the present and previous studies, foliar Zn spray with or without pesticides are crucial to grain Zn enrichment [4,13,15–17]. In this scenario, grain Zn was more regulated by grain internal factors [11,31,32].

Previous studies have found that there is significant within-seed control over Zn entering the seed endosperm [31]. Two barriers of Zn transport into wheat grains may exist between the stem tissue rachis and the grain, and between the maternal and filial tissues in the grain [31,32]. The latter barrier often occurs in the crease regions, seed coat, and aleurone layer of grain, exhibiting a huge gap of Zn concentration against the endosperm [11]. Under the present conditions, the abundant Zn accumulation in bran (which mainly consists of seed coat and aleurone) were less affected by yield differences between the two years (Figure 3), indicating sufficient Zn pools in these maternal tissues of wheat with foliar Zn applications. The absence of correlation between Zn concentration and its binding ligands (mainly phytate and protein) in bran further endorsed the possible saturation of Zn in this tissue (Figures 5 and 6). Many studies have also found that Zn is easily accumulated in the crease regions and outer layers of wheat grain, especially with foliar application of Zn fertilizer [11,23,33]. Thus, more Zn transport from these tissues to the starchy endosperm (flour fractions) would be

meaningful and expected. Increasing Zn supply can improve the Zn levels in both whole-grain and endosperm tissues [11], which was also observed in this study by foliar Zn spray with or without pesticides. However, the flour Zn concentration and bioavailability are still below the required targets (Figures 3 and 7) [1,4]. The strong and positive correlations between Zn and its ligands (preferably protein) in flour fraction by foliar Zn sprays (with and without pesticides) implied Zn concentrations could be further enhanced in coordination with plant N nutritional status (Figure 6) [11,23,34].

In addition, although grain Zn concentrations differed because of yield variations, grain Zn biofortification was achieved and exceeded the breeding target in plants with both lower and higher yield capacity. Considering the increasing food demand and widespread Zn malnutrition issues, increasing grain Zn concentration in wheat with high yield capacity would be promising.

## 5. Conclusions

In summary, the effectiveness of combined foliar application of Zn and pesticides has been validated in terms of Zn enrichment and bioavailability in grain and its milling fractions. Compared to the nil Zn spray, foliar application of Zn alone or in combination with pesticides substantially increased Zn concentrations and bioavailability in the whole-grain, bran, and flour, irrespective of spray timing. Yield variation between years also exerted impacts on grain Zn status, while such a relationship was uncoupled by foliar spray of Zn or plus pesticides, and absent from the plants without Zn spray. Nonetheless, grain Zn concentrations exceeded the biofortification target in both low- and high-yield plants with either Zn spray alone or combined with pesticides. Together with the enhanced Zn bioavailability in grain, bran, and flour, the present study clearly demonstrated that foliar Zn application can be safely applied with commonly used fungicides and insecticides either at anthesis or milk stage to enrich Zn of the whole-grain and its milling fractions.

**Author Contributions:** Conceptualization, X.T., P.N., and S.W.; validation, P.N. and S.W.; formal analysis, P.N. and S.W.; investigation, S.W., P.F., X.Z., J.D., and J.S.; writing—original draft preparation, P.N.; writing—review and editing, X.T.; supervision, X.T.; project administration, J.S.; funding acquisition, X.T. and P.N.

**Funding:** This research was funded by the National Natural Science Foundation of China, grant number 31801929 and 31672233.

**Acknowledgments:** The authors would like to thank Juan Chen, Wenling Chen, and Song Wang for their assistance with the field work.

**Conflicts of Interest:** The authors declare no conflict of interest.

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