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Glycine-chelated zinc rather than glycine-mixed zinc has lower foliar phytotoxicity than zinc sulfate and enhances zinc biofortification in waxy corn

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ABSTRACT

To determine whether high spraying concentrations of Zn sources increase the Zn concentration in waxy corn (*Zea mays* L. var. ceratina Kulesh) seeds without compromising agronomic performance, field experiments were conducted between 2018 and 2020. Excess $ZnSO_4$ application caused foliar burn, barren ear tip, and grain yield loss. ZnEDTA and Glycine-chelated Zn (ZnGly) caused less foliar burn, but Glycine-mixed Zn caused more foliar burn than $ZnSO_4$. The seed Zn concentration increased with spraying Zn concentration. ZnEDTA ($\leq$0.8%) had a higher threshold concentration than ZnGly ($\leq$0.4%). Nevertheless, Zn biofortification efficacy did not significantly differ between 0.4% ZnGly and 0.8% ZnEDTA, and the grain Zn recovery rate of 0.4% ZnGly was much higher than that of 0.8% ZnEDTA. Additionally, dual-isotope labelling tests confirmed that ^{15}N-glycine and ^{68}Zn in ZnGly interacted. In the future, chelating technology is essential for developing new Zn fertilizers to optimize Zn biofortification efficacy.

1. Introduction

Globally, Zinc (Zn) deficiency is associated with a low concentration of Zn in common cereal crops (Wessells & Brown, 2012; Yadav et al., 2020). Consequently, the production of Zn-fortified grain has been a shared objective of crop breeders and agrochemists. Waxy corn (*Zea mays* L. var. ceratina Kulesh) is consumed as both starch source and vegetable. Although waxy corn originated in China, it is being consumed globally for its flavorful characteristics and high nutritional value (Schwartz & Whistler, 2009). In addition, the improved health benefits of Zn in Zn-fortified waxy corn appeal to consumers and farmers (Akhtar et al., 2018; Yadav et al., 2020). Hence, cultivating Zn-rich varieties is an approach to improve grain Zn concentration. Regardless of transgenic or non-transgenic breeding, Zn-enriched corn varieties have not been planted extensively as they have long breeding cycles and poor adaptability, in addition to the enduring concerns regarding the safety of genetically modified foods (Agarwal et al., 2018; Bouis and Saltzman, 2017).

Zn-fortified waxy corn can also be produced through soil amendment with Zn fertilizer. However, the grain Zn concentration of cultivars grown in Zn-sufficient soils are inadequate to meet human nutritional requirements. This inadequacy could be because soil Zn bioavailability for uptake by roots is considerably influenced by soil pH, moisture, organic matter content, and antagonistic cations, regardless of soil amendment with Zn fertilizer (Montalvo et al., 2016). Furthermore, soil Zn application decreases Zn use efficiency (ZnUE), leading to considerable Zn fertilizer losses and potentially posing risks of soil contamination (Wang et al., 2012).

Foliar Zn fertilizer application is a rapid strategy to produce Zn-fortified grain, independent of crop varieties and soil types (Cakmak, 2008; Zou et al., 2012). Foliar Zn fertilizer application also enhances ZnUE (Wang et al., 2012). Zinc sulfate ($ZnSO_4$) is the most used form of Zn fertilizer (Montalvo et al., 2016). The Zn concentration in the edible parts of wheat (Dapkekar et al., 2018; Zhang et al., 2012) and green bean

Abbreviations: DTPA, Diethylenetriaminepentaacetic acid; EC, Electrical conductivity; NUE, *N*-use efficiency; ZnEDTA, ethylenediaminetetraacetic acid disodium zinc salt tetrahydrate; ZnUE, Zn use efficiency; ZnGly, glycine-chelated Zn; nZnOs, zinc oxide nanoparticles.

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(Almeida et al., 2020) has been steadily increased with an increase in foliar $ZnSO_4$ spraying concentration. However, excess foliar Zn application could cause foliar burn, nutrient imbalances, yield loss at harvest, and growth inhibition (Drissi et al., 2015; Golden et al., 2016). Consequently, zinc sulfate heptahydrate ($ZnSO_4{\cdot}7H_2O$) is often used at low spraying concentrations, that is, < 0.5% (w v^{-1}).

Another widely used Zn fertilizer is ethylenediaminetetraacetic acid disodium zinc salt tetrahydrate (ZnEDTA) (Montalvo et al., 2016). Golden et al. (2016) reported that the severity of foliar burn after ZnEDTA application was lower than that after $ZnSO_4$ application, although the basic physicochemical properties of Zn fertilizers and the corn Zn concentrations were not specified. Both acid damage and salt damage could cause foliar burn. Therefore, ZnEDTA has a higher spray concentration of safety threshold than $ZnSO_4$, possibly because the pH and electrical conductivity (EC) of ZnEDTA are safer for leaf than those of $ZnSO_4$. To the best of our knowledge, no study has explored the mechanisms of reducing $ZnSO_4$-induced foliar burn.

Natural amino acids, usually glycine (Gly), are used as "green" alternatives to artificial chelating agents such as EDTA (Souri & Hatamian, 2019). Chelated fertilizers based on natural amino acids are essentially non-toxic to living things, making them highly suitable for sustainable agriculture (Dolev et al., 2020; Souri & Hatamian, 2019). Glycine-chelated Zn (ZnGly) exhibits Zn biofortification superior to both $ZnSO_4$ and ZnEDTA (Ghasemi et al., 2013; Tabesh et al., 2020; Wei et al., 2012). Shen et al. (2017) reported that the foliar application of a non-chelating mixture of Gly plus Zn (Zn-Gly) promoted foliar Zn uptake in Chinese cabbage. However, no Zn-Gly mixture controls have been used in previous ZnGly biofortification studies to rule out any possible synergy between Zn and Gly. Currently, zinc oxide nanoparticles (nZnOs) are a research hotspot in agriculture (Sturikova et al., 2018). However, nZnOs have poor solubility and therefore seem unsuitable for foliar application. Nevertheless, foliar nZnOs spraying at low concentrations enhances Zn uptake by crops (Adrees et al., 2021; Rossi et al., 2019). Previous studies did not use zinc oxide (ZnO) as a control to disclose any nanoparticle-specific effects of nZnOs.

The Zn biofortification effects of various Zn spray concentrations have seldom been reported except for those of $ZnSO_4$. Earlier studies only conducted tests based on common application concentrations to compare the relative efficacies of various Zn sources. Whether chelated Zn fertilizers can further improve grain Zn concentration while concomitantly relieving $ZnSO_4$-induced foliar burn requires a comprehensive study. Field-scale studies are mandatory to maximize seed Zn concentrations without compromising agronomic performance. We examined the effects of normal to high spraying concentrations of foliar $ZnSO_4$, ZnEDTA, Gly-chelated zinc (ZnGly), Gly-mixed zinc (Zn-Gly), ZnO, and nZnOs spray on foliage health, photosynthetic performance, yield and yield components, and element level changes.

The specific objectives of the present study were to (1) identify and determine the synergy between Zn and Gly in ZnGly and Zn-Gly, (2) distinguish the nanoparticle-specific effects by comparing the effects of nZnOs and ZnO, (3) clarify the relationships among foliar burn, pH, and EC associated with foliar Zn fertilizers, (4) establish foliar Zn absorptivity and mobility to account for the differences in efficacy among Zn sources, and (5) accurately evaluate ZnUE via isotopic labeling. The present study results could facilitate the optimization of Zn-biofortified grain production via foliar Zn fertilizer application.

2. Materials and methods

2.1. Experimental conditions and management measures

Field trials were conducted in the experimental station of the Zhejiang Academy of Agricultural Sciences (30°19 N, 120°12 E) in Hang Zhou, Zhejiang Province, China. The region has a typical subtropical monsoon climate. The monthly average temperature, precipitation, and sunshine hour data during the growing season were compiled by the Hangzhou Meteorological Service Center and are listed in Table S1 of the Supplementary Material. The soil is classified as a Hydragric Anthrosol according to WRB classification (World Reference Base for Soil Resources, 2015). In 2018, five topsoil layer (0–30 cm) samples were randomly collected for chemical analyses. The soil pH was 6.31 and the soil organic matter concentration was 29.43 g kg^{-1}. The alkali-hydrolyzable nitrogen (N), Olsen-available phosphorus (P), and NH_4OAc-available potassium (K) levels were 137.37, 22.34, and 89.91 mg kg^{-1}, respectively; the diethylenetriaminepentaacetic acid (DTPA)-Zn and DTPA-Fe levels were 2.06 and 138.18 mg kg^{-1}, respectively.

The sweet-waxy corn cultivars tested were the normal-yield Meiyu8, used as a control variety, and the high-yielding Kenuo2, registered and released in 2016 by the Zhejiang Provincial Crop Variety Appraisal Committee. The seeds were sowed in an N—S orientation on 1.1 m wide ridges adjacent to a 0.2 m wide drain. Two rows of corn seeds were planted per ridge, and the rows were separated by 0.55 m. Total N (180 kg N ha^{-1} as coated urea), P (120 kg P_2O_5 ha^{-1} as triple superphosphate), and K (225 kg K_2O ha^{-1} as potassium chloride) were incorporated as base fertilizer in all treatments. All other field management measures excluding foliar fertilizer application were consistent with those of the standard practices of professional field managers. Some field operations are listed in Table S1.

2.2. Preparation of foliar Zn fertilizers

The Zn fertilizers used were $ZnSO_4{\cdot}7H_2O$ (22.74% Zn w w^{-1}), ZnEDTA (16.37% Zn w w^{-1}), nZnOs (80.36% Zn w w^{-1}; 50 ± 10 nm), and ZnO powder (80.36% Zn w w^{-1}). The Zn chelators used were Gly and ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA). All reagents (guaranteed reagent, GR) were purchased from Shanghai Aladdin Biochemical Technology Co. Ltd. (Shanghai, China). Ten percent (w v^{-1}) Zn-Gly was prepared by adding Gly (1:2.5 Zn:Gly molar ratio) to 10% $ZnSO_4$ (as $ZnSO_4{\cdot}7H_2O$ [w v^{-1}]). Ten percent ZnGly was prepared by heating 10% Zn-Gly solution at 70℃ for 1.5 h. Tween-80 (polyoxyethylene sorbitan fatty acid esters, 0.01% (v v^{-1})) was added to the fertilizer solutions as a surfactant.

The method described by Wu et al. (2010) was followed for preparing 0.1% $^{68}ZnSO_4$ (as $ZnSO_4{\cdot}7H_2O$ [w v^{-1}]) with ^{68}ZnO. The isotope enrichments in ^{68}ZnO solid powder (Trace Sciences International, Toronto, Canada) were as follows: 98.33% ^{68}Zn, 0.76% ^{64}Zn, 0.61% ^{66}Zn, 0.27% ^{67}Zn, and 0.03% ^{70}Zn. The Zn isotope abundances in non-enriched $ZnSO_4$ were as follows: 18.80% ^{68}Zn, 48.68% ^{64}Zn, 27.86% ^{66}Zn, 4.07% ^{67}Zn, and 0.59 % ^{70}Zn. For preparing 0.1% $^{68}ZnEDTA$ (EDTA-chelated $^{68}ZnSO_4$), EDTA was added at a 1:1 Zn:EDTA molar ratio to 0.1% $^{68}ZnSO_4$ (w v^{-1}) before adding $^{68}ZnSO_4$. For preparing 0.1% ^{15}N-^{68}Zn-Zn-Gly solution, ^{15}N-glycine (98.0 atom% ^{15}N; Sigma-Aldrich LLC, Schnelldorf, Germany) was added at a 1:2.5 Zn:Gly molar ratio to 0.1% $^{68}ZnSO_4$ (w v^{-1}). For preparing 0.1% ^{15}N-^{68}Zn-ZnGly (w v^{-1}), isotope-labeled Zn-Gly solution was heated at 70 ℃ for 1.5 h. Tween-80 (0.01% [v v^{-1}]) was added to isotope-labeled fertilizer solutions as a surfactant.

2.3. Foliar Zn fertilizer application

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2.3.1. Efficacy of Zn sources at different spraying concentrations in 2018

$ZnSO_4$, ZnEDTA, ZnGly, and Zn-Gly were tested on Kenuo2 in 2018 using a randomized complete block design with three replicates. Ten percent (w v^{-1}) 10% $ZnSO_4$ (as $ZnSO_4{\cdot}7H_2O$ [w v^{-1}]) was diluted to 0.1%, 0.2%, 0.4%, 0.8%, 1.6%, 3.2%, and 6.4%. ZnEDTA, ZnGly, and Zn-Gly were set at the same Zn^{2+} concentration gradient as the $ZnSO_4$ treatment; the Zn spraying concentration of all Zn sources presented in the text is the concentration after transforming. Water was used the control (CK). The pH and EC of different Zn formulations are listed in Table S2 of the Supplementary Material.

Protective rows surrounded the rectangular experimental field. The field was divided into 87 plots. Each plot contained 30 plants and was surrounded by a guard ridge. After the silking period, the solutions were sprayed using a pressure sprayer (702C, LvTai, Taizhou Luqiao Lvtai Sprayer Factory, Taizhou, China) after 17:00 h on rainless, windless days. A total of three sprays were performed at 5 d intervals, and the spraying quantity of each spray was 700 L ha^{-1}. However, the 1.6%, 3.2%, and 6.4% Zn concentrations were only applied once because all Zn concentrations > 0.8% caused foliar burn.

2.3.2. Efficacy of different Zn formulations in 2019 and 2020

Here, 0.4% ZnGly, 0.4% $ZnSO_4$, and 0.8% ZnEDTA were selected based on Zn biofortification effects, foliar burn, and yield levels determined in the 2018 trial. ZnO and ZnOs were used at 0.1% (the same Zn^{2+} concentration gradient as the $ZnSO_4$ treatment) spraying concentration to detect nanoparticle-specific effects of nZnOs. ZnO and nZnOs were only slightly soluble in water. The five Zn formulae and CK were tested on the Kenuo2 and Meiyu8 cultivars in 2019 and 2020, based on a randomized complete block design with three replicates.

2.3.3. Dual-isotope labelling tests: Zn mobility in the ear leaf and Zn transfer to the seeds

Kenuo2 was selected as the experimental cultivar because the 2019 field tests revealed its relatively high ear yield and seed Zn concentration. Before the isotope assays, plants that consistently grew after the silking period in the 2020 field test were sprayed several times with pure water. The following isotope-labeled fertilizers were applied: 0.1% $^{68}ZnSO_4$, 0.1% ^{15}N-^{68}Zn-Zn-Gly, 0.1% ^{15}N-^{68}Zn-ZnGly, 0.1% ^{68}ZnEDTA, and ^{15}N-Gly. The dosage of ^{15}N-Gly was similar to that of Gly in 0.1% ^{15}N-^{68}Zn-ZnGly.

The leaves were marked 40 cm from the ear leaf tip using a marker pen. The marked parts were dipped in fertilizer solutions for 30 s. After 48 h, the marked leaves were excised and cut into sections. The first section was cut 20 cm from the leaf tip. The second was cut between 20 and 40 cm from the leaf tip. Additionally, four sections of length 5 cm were prepared. Each treatment had three replicates, and each replicate comprised of three plants.

Another experiment was conducted to trace fertilizer translocation to the grain and evaluate ZnUE. A total of three sprays were performed at 5-d intervals, and the spraying quantity of each spray was 70 mL of isotope-labeled fertilizer solution. The sprays were performed after the silking period, using a small pressure sprayer (100PET; Shuaikang Suye Factory, Suzhou, China). Each treatment included three replicates, and each replicate comprised three plants. Unlabeled 0.1% $ZnSO_4$ was used as the reference.

2.4. Leaf health assessment

Ear leaf health status was assessed by one researcher to ensure consistent evaluation. Five days after the foliar application, healthy leaf areas unaffected by fertilizer-induced damage were visually estimated as a percentage of the total leaf area (Golden et al., 2016). The net photosynthesis rate (*Pn*) and Soil Plant Analysis Development (SPAD) values of the right side of the ear leaf bases were measured using LI-6400 XT portable photosynthesis system (LI-COR Inc., Lincoln, NE, USA) and a SPAD meter (Minolta SPAD-502, Tokyo, Japan), respectively. Five days after the last foliar application, the measurement was performed within 2.5 h beginning at 9:30 to minimize the variation due to changes in time of day.

2.5. Yield, sampling, and analytical methods

The ears of waxy corn were manually collected each year at the optimal commodity harvest dates, assessed by an expert from the experimental station. Fresh spike weights (commodity) were determined. Ten ears per treatment were heat-treated for 30 min at 105 °C and then dried at 65 °C to a constant weight for analyzing their separate yield components. Three representative dried ears per treatment were selected for elemental analysis. Dried seed samples were pulverized in an agate mortar (Model MM301; Retsch GmbH, Haan, Germany) and packed in plastic bags.

N concentration was measured using the alkaline hydrolysis diffusion method (Wang et al., 2012). Seed samples were digested in 20 mL of nitric acid (HNO_3, GR), perchloric acid ($HClO_4$; GR), and hydrogen peroxide (H_2O_2; 5:2:1 [v v^{-1}]) to measure the other elements. The digests were cooled to 25 °C and diluted to 50 mL with double-distilled deionized water. Element concentrations were determined using an inductively coupled plasma-atomic emission spectrometer (Thermo 6300; Thermo Fisher Scientific, Waltham, MA, USA). The element concentrations of the reference material (polished rice powder, GBW [E] 080684) and blank controls were also measured.

To quantify the foliar Zn fertilizer utilization efficiency, the grain Zn recovery rate—defined as the percentage of Zn ions that were apparently translocated from the fertilizer to the seeds at harvest—was calculated as follows (Wang et al., 2012):

$$Grain\ Zn\ recovery\ rate(\%) = \frac{C_t \times W_t - C_0 \times W_0}{F} \times 100 \quad (1)$$

where, C_t and C_0 represent the Zn concentration (mg kg^{-1}) in seeds treated with or without the foliar Zn fertilizer, respectively; *Wt* and W_0 represent the seeds weight (kg ha^{-1}) treated with or without the foliar Zn fertilizer, respectively; and *F* is the total Zn ion input (mg ha^{-1}) of the foliar Zn fertilizer.

To standardize the changes in the ratio of each element to Zn in the Zn-fortified seeds under various treatments, the standardized element: Zn ratio was calculated as follows:

$$Standardized\ element : Zn\ ratio = \frac{T_e/C_t}{T_0/C_0} \quad (2)$$

where, C_t and C_0 represent the Zn concentration (mg kg^{-1}) in seeds treated with or without the foliar Zn fertilizer, respectively; *Te* and T_0 represent the element concentration (mg kg^{-1}) in seeds treated with or without the foliar Zn fertilizer, respectively.

2.6. Isotope-labeled samples and analytical methods

Each replicate contained leaf sections or seeds from all plants. ^{15}N and ^{68}Zn enrichments were determined using Tracer MAT-271 (Finnigan MAT; Thermo Fisher Scientific, Waltham, MA, USA) and an inductively coupled plasma mass spectrometer (Model 7500a; Agilent Technologies, Santa Clara, CA, USA), respectively, at the Analysis and Testing Center of Zhejiang University. The isotope-labeled dipped ear leaf sections were washed several times with water, 50 mM calcium chloride, and double-deionized water. The seeds were collected at harvest and analyzed using the abovementioned non-isotopic test methods.

2.7. Statistical analysis

Data are expressed as mean ± SD; they were analyzed using STATISTICA v. 5.5 (TIBCO Software Inc., Palo Alto, CA, USA) and Microsoft Excel 2016 (Microsoft Corp., Redmond, WA, USA). Shapiro–Wilk test was used to assess variable normality among treatments; it was followed by one-way analysis of variance and Tukey's honestly significant difference test. The significance level was 0.05.

3. Results

3.1. Effects of Zn formulations at different concentrations on Kenuo2 in 2018

3.1.1. Agronomic performance

Fertilizer-induced foliar burn was manifested as visible yellow mottles, streaks, and curled leaves (Fig. 1a). None of the Zn sources was phytotoxic at the 0.1% and 0.2% spraying concentrations (Fig. 1b). At the 0.4% and 0.8% spraying concentrations, all Zn sources excluding ZnEDTA caused visible foliar phytotoxicity. At application concentrations > 0.8%, all fertilizers caused substantial foliage damage; the order of severity was CK < ZnEDTA < ZnGly < $ZnSO_4$ < Zn-Gly. Both chelated Zn sources induced less foliar burn than excess $ZnSO_4$. Furthermore, ZnEDTA had a safe threshold concentration (≤0.8%) that was at least 1-fold greater than that of other Zn formulations.

Barren ear tips always appeared at spraying concentrations > 0.4% (Fig. 1c), and negatively affected waxy corn yield (Fig. 1d). Fresh spike and dry seed weights decreased with an increase in Zn concentration. The fresh spike weights did not significantly differ among the Zn fertilizer types at spraying concentrations ≤ 0.4%. However, these weights were always lower for Zn-Gly and ZnEDTA than for ZnGly at spraying concentrations > 0.4%. The dry seed weights increased in the order Zn-Gly < ZnEDTA and $ZnSO_4$ < ZnGly, and were usually significantly lower for Zn-Gly than for ZnGly at spraying concentrations > 0.2%.

The SPAD value and *Pn* decreased with an increase in spraying rate (Fig. S1). At spraying concentrations > 0.4%, the SPAD value and *Pn* decreased in the order CK < ZnEDTA < ZnGly < $ZnSO_4$ < Zn-Gly. For all Zn sources, both seed number and hundred-grain weight slightly decreased with an increase in the spraying concentration (Fig. S2). Nonetheless, the seed numbers did not significantly differ among the Zn treatments. The hundred-grain weight was usually the lowest for Zn-Gly at spraying rates > 0.2% and the highest for ZnGly at spraying concentrations > 0.8%.

3.1.2. Element concentrations in seeds

The grain Zn concentration increased with an increase in spraying concentration in the 0.1%–0.8% and 1.6%–6.4% ranges (Fig. 2a). At the 0.1% and 0.2% spraying concentrations, the grain Zn concentrations were similar for all Zn sources. At spraying concentrations > 0.2%, ZnGly achieved the highest grain Zn concentrations. In particular, the grain Zn concentrations were significantly higher in the seeds of plants exposed to ZnGly than in those of plants exposed to ZnEDTA. The grain Zn recovery rate decreased with an increase in Zn concentration for all Zn sources. At spraying concentrations > 0.1%, ZnGly achieved the highest grain Zn recovery rate. The grain Zn recovery rate was < 1% when Zn spraying concentration of all Zn sources exceeded 0.8%.

With an increase in the spraying concentration, all standardized

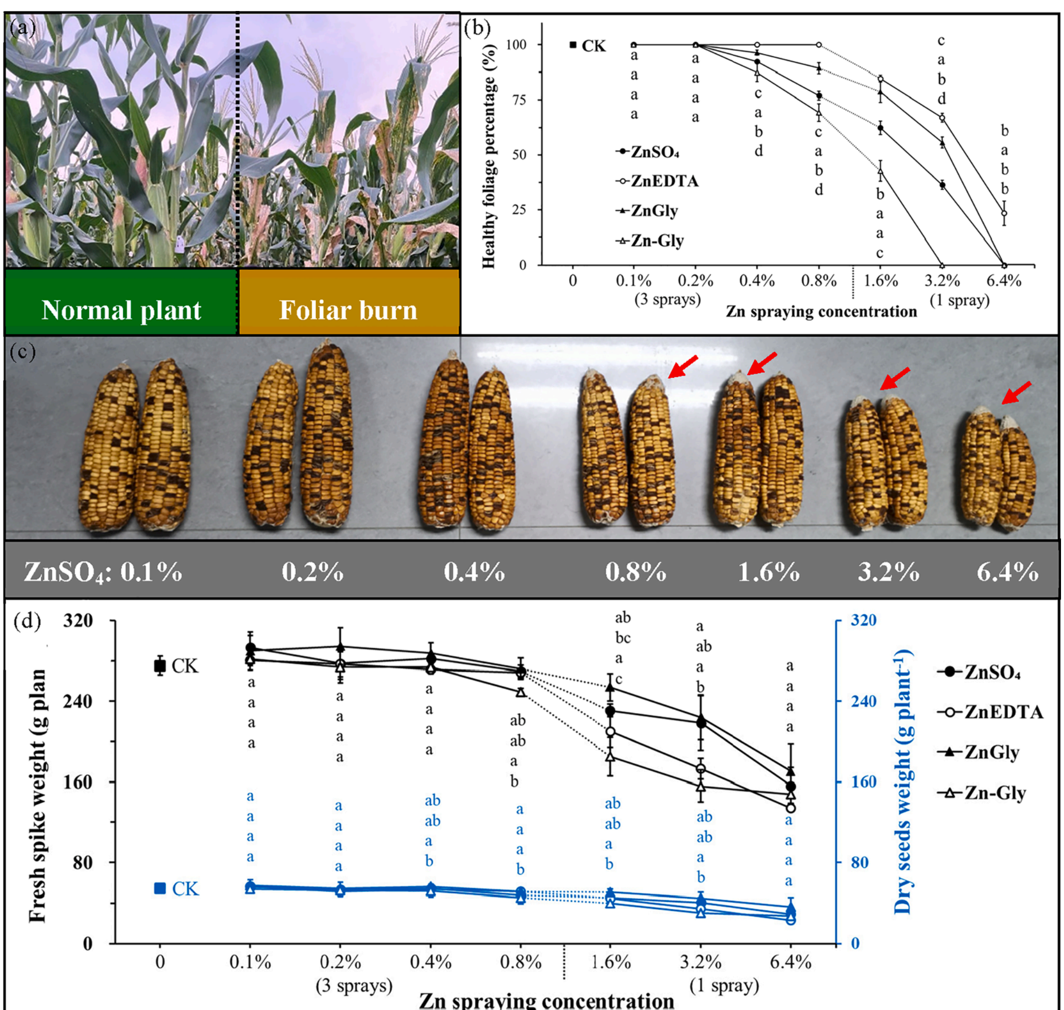

Fig 1. (a) $ZnSO_4$-induced foliar injury in the field; (b) percent healthy foliage; (c) barren ear tip; (d) fresh spike weight and dry seed weight. Lowercase letters from top to bottom at the same Zn spraying concentration are in the same order as in the legend. Different lowercase letters indicate significant difference at the 5% significance level. ZnEDTA, ZnGly, and Zn-Gly were set at the same Zn^{2+} concentration gradient as the $ZnSO_4$ treatment.

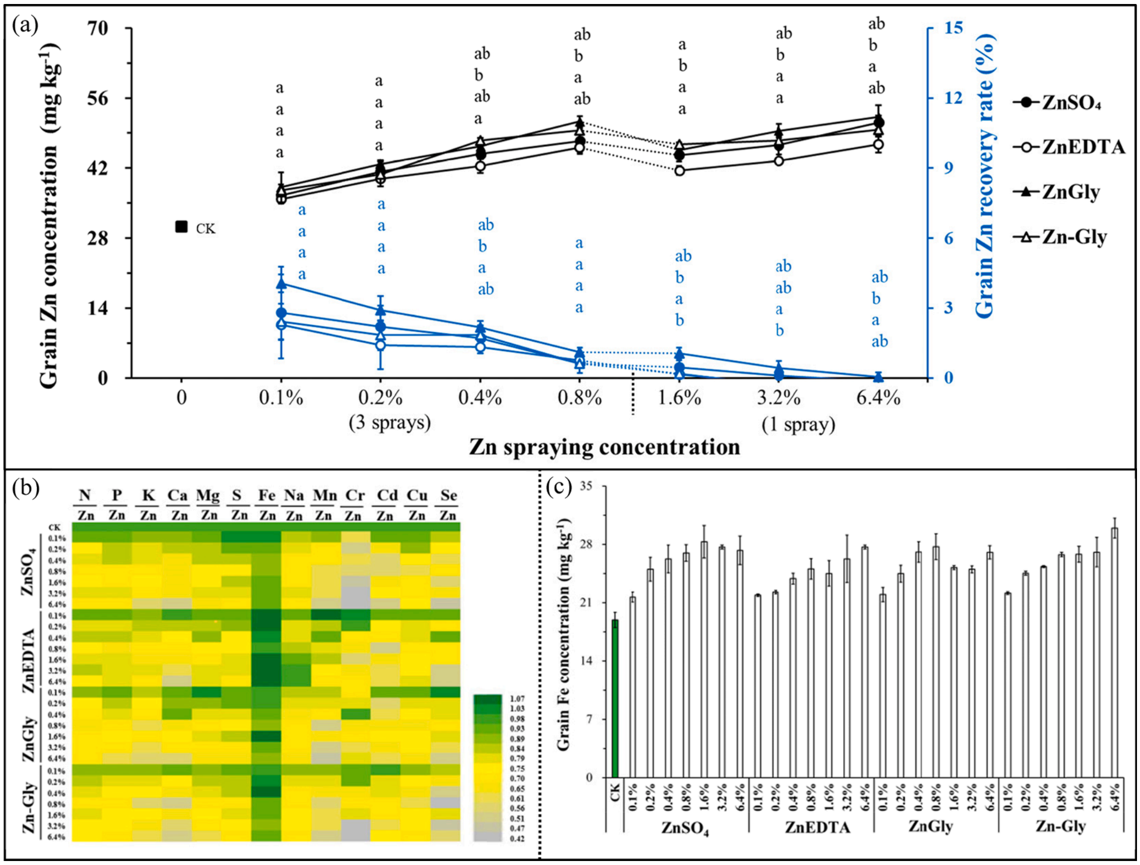

Fig 2. (a) Zn concentration in the grain seed and grain Zn recovery rate; (b) Standardized element:Zn ratio in the grain seed under different treatments; (c) Fe concentration in dry grain seed. Lowercase letters from top to bottom at the same Zn spraying concentration are in the same order as in the legend. Different letters indicate a significant difference at the 5% level. ZnEDTA, ZnGly, and Zn-Gly were set at the same Zn^{2+} concentration gradient as the $ZnSO_4$ treatment.

element:Zn ratios, excluding Fe:Zn, decreased (Fig. 2b). The lowest standardized ratios declined from 1 for CK to < 0.5. At high Zn spraying concentrations, there were imbalances in most metal cations. The seed Fe concentration increased with an increase in the Zn spraying concentration (Fig. 2c).

Based on agronomic and Zn-fortified performance, 0.4% ZnGly, 1.0% ZnEDTA, and 0.4% $ZnSO_4$ were selected and tested repeatedly in the two waxy corn varieties Meiyu8 and Kenuo2 in the subsequent years.

3.2. Effects of different Zn formulations on Meiyu8 and Kenuo2 in 2019 and 2020

The fertilizers 0.4% $ZnSO_4$ and 0.4% ZnGly caused slight foliar burn in Meiyu8 and Kenuo2. However, the damage caused by 0.4% ZnGly was less severe than that caused by 0.4% $ZnSO_4$ (Fig. 1b). In both 2019 and 2020, there were no significant differences among Zn fertilization treatments and CK in terms of SPAD value, ear leaf net photosynthetic rate, seed number per ear, and hundred-grain weight (data not shown).

There were no significant differences among the fertilization treatments in terms of fresh spike weight in both cultivars and years (Fig. 3). Compared with CK, all fertilization treatments significantly improved seed Fe concentration. Neither 0.1% ZnO nor 0.1% nZnOs increased the seed Fe concentration to levels similar to those of the other Zn formulations. There were no differences between ZnO and nZnOs in terms of seed Fe concentration or fresh spike weight.

Compared with CK, all fertilization treatments significantly improved the seed Zn concentration (Fig. 4). The 0.1% ZnO and 0.1% nZnOs treatments achieved the highest grain Zn recovery rate. There were no differences between ZnO and nZnOs with regard to seed Zn concentration or grain Zn recovery rate. Nevertheless, the ZnO and nZnOs treatments increased the seed Zn concentrations to a relatively lower degree than the other Zn formulations. In 2019, the grain Zn recovery rate for 0.8% ZnEDTA was only approximately half of that for 0.4% $ZnSO_4$ and 0.4% ZnGly. Therefore, a repeatability test was conducted in 2020 using only 0.4% $ZnSO_4$ and 0.4% ZnGly. In the 2019 and 2020 tests, the seed Zn concentration was higher under the 0.4% ZnGly treatment than that under 0.4% $ZnSO_4$ treatment.

3.3. Isotope labeling experiments with Kenuo2 in 2020

The ^{68}Zn concentrations in all leaf sections (excluding 0–20 cm) decreased in the order Zn-Gly and ZnEDTA > $ZnSO_4$ > ZnGly > no ^{68}Zn treatment (Gly) (Fig. 5), whereas the ^{15}N concentrations in leaf sections decreased in the order Gly > Zn-Gly > ZnGly > no ^{15}N treatments ($ZnSO_4$ and ZnEDTA). Zn-Gly applied to the leaves was more mobile than ZnGly, indicating that chelation affects the mobility of Zn and Gly in the leaves.

The ^{15}N concentrations in the seeds under the $ZnSO_4$ and ZnEDTA treatments and the ^{68}Zn concentrations in the seeds under the Gly treatment were considered the control values (Fig. 6a and 6b). The ^{15}N-Gly and ^{68}Zn fertilizers significantly improved the relative seed ^{15}N and ^{68}Zn concentrations, respectively. Compared with the Gly treatment, the unlabeled $ZnSO_4$ treatment increased the ^{68}Zn concentration; nonetheless, it was lower than that under the treatments containing ^{68}Zn. The ^{68}Zn: total Zn ratio was lower under the unlabeled $ZnSO_4$ treatment than under the Gly treatment. The ^{68}Zn concentrations did not differ significantly among the ^{68}Zn fertilizer treatments. In contrast, the ^{15}N concentrations significantly differed among ^{15}N fertilizers in the order Gly > Zn-Gly > ZnGly. Furthermore, all ^{68}Zn fertilizers and unlabeled $ZnSO_4$ significantly enhanced the total seed Zn concentration and to a greater

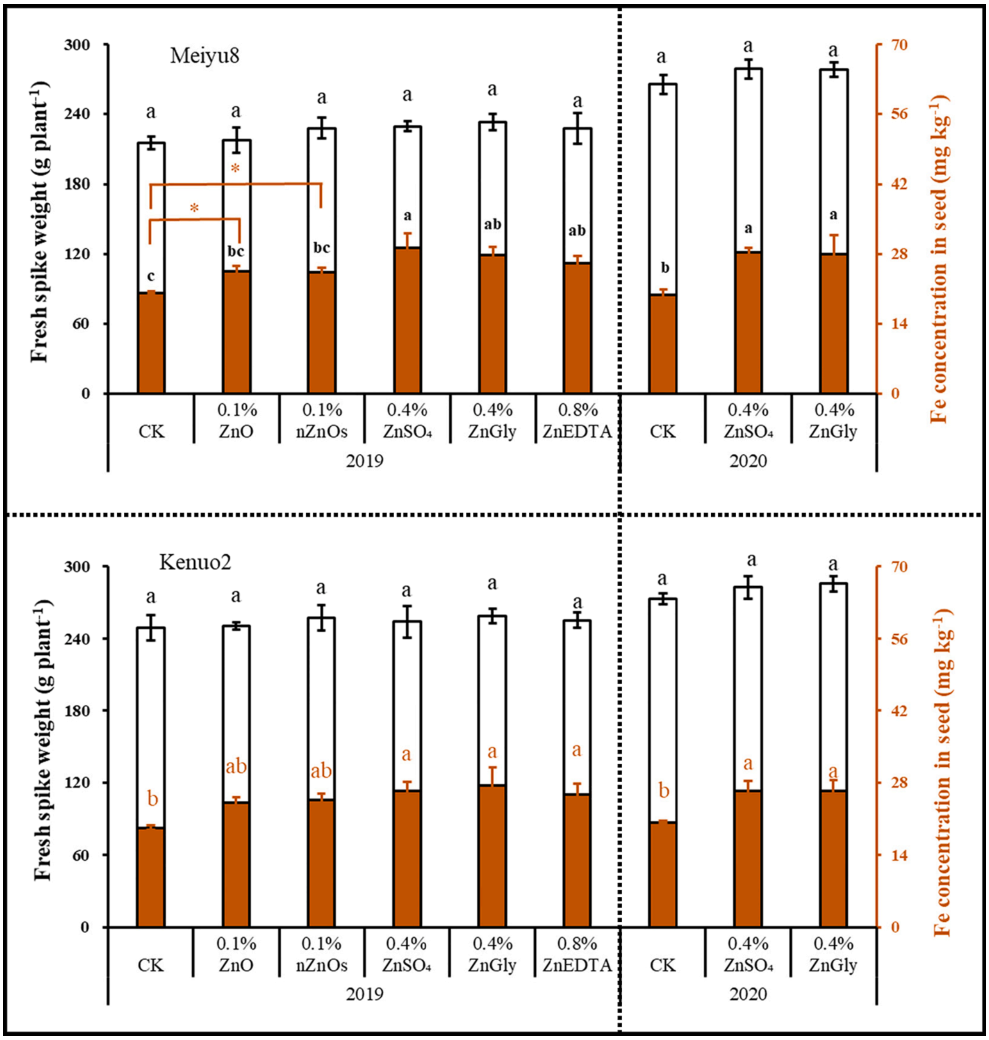

Fig 3. Effects of spraying different Zn formulations on fresh spike weight and Fe concentration in Meiyu8 and Kenuo2 dry seeds in 2019 and 2020. Different small letters under the same year are significantly different at the 5% significance level. * indicates significant differences ($p < 0.05$). ZnEDTA, ZnGly, and Zn-Gly were set at the same Zn^{2+} concentration gradient as the $ZnSO_4$ treatment.

extent than ^{68}Zn concentration. Under all Zn treatments, the grain Zn recovery rates were considerably higher than grain ^{68}Zn recovery rates (Fig. 6c).

4. Discussion

4.1. Variability in fertilizer-induced foliar burn

Here, when the spraying Zn concentrations were > 0.4% (w v^{-1}), the most widely used $ZnSO_4$ fertilizer not only caused foliar burn and impaired photosynthesis but also created imbalances in the ratios of Zn to other metal cations, hindered normal grain filling, caused barren ear tips, and decreased grain yield. Simple non-chelating addition of Gly to $ZnSO_4$ (Zn-Gly treatment) aggravated phytotoxicity. Excess Zn^{2+} is associated with $ZnSO_4$-induced foliar damage (Qiao et al., 2014). The results of a short-term absorption experiment revealed that Zn-Gly increased both Zn^{2+} and Gly uptake in the ear leaves. Furthermore, the Zn-Gly solution had a lower pH and higher EC than the $ZnSO_4$ solution. It is assumed that crop leaf damage severity increases with a decrease in pH and an increase in EC. Therefore, the $ZnSO_4{\cdot}7H_2O$ application concentration is generally in the range of 0.1–0.5% (w v^{-1}) to avoid foliar salt damage, and the pH values of foliar Zn fertilizers are typically adjusted to 5–8 to prevent acid injury. However, the present study showed that pH < 5 and/or Zn concentration > 0.5% did not always cause foliar burn (Table S2 and Fig. 1). The pH of ZnGly was lower than that of $ZnSO_4$, and the EC of ZnEDTA was higher than that of $ZnSO_4$. Nevertheless, both chelated fertilizers (ZnGly and ZnEDTA) reduced foliar injuries compared with $ZnSO_4$. Therefore, Zn type—rather than pH or EC—influenced foliar burn severity.

EDTA is a stronger Zn chelator than Gly (Dolev et al., 2020; Marešová et al., 2012). The concentrations of free Zn ions in the foliar fertilizers were in the order of CK < ZnEDTA < ZnGly < $ZnSO_4$; the degree of foliage burn exhibited a similar trend (Fig. 1). ZnEDTA was more readily absorbed and more mobile than $ZnSO_4$ in the ear leaves, which contradicted the that previous assumption. As it resulted in higher ^{68}Zn levels, the ZnEDTA treatment should have caused more severe foliar burn than the $ZnSO_4$ treatment if the Zn form in the ear leaves was toxic Zn^{2+}. Furthermore, yellow crinkles on the leaf blade emerged only under ZnEDTA treatments. Hence, ZnEDTA provided Zn to the ear leaves in a form different from that provided by $ZnSO_4$. Zn radioisotope tracers and synchrotron-based X-ray absorption near-edge structures have demonstrated that intact ZnEDTA chelate is immediately absorbed into wheat seedling leaves (Doolette et al., 2018). Therefore, the less leaf burn caused by ZnEDTA was probably due to the reduced Zn ion concentrations in foliar fertilizers and the relative nontoxicity of the ZnEDTA complex compared with the toxicity caused by Zn ions in the leaves. ZnGly could have been absorbed through the leaf in an intact chelate form because comparatively less ^{15}N was absorbed from ZnGly

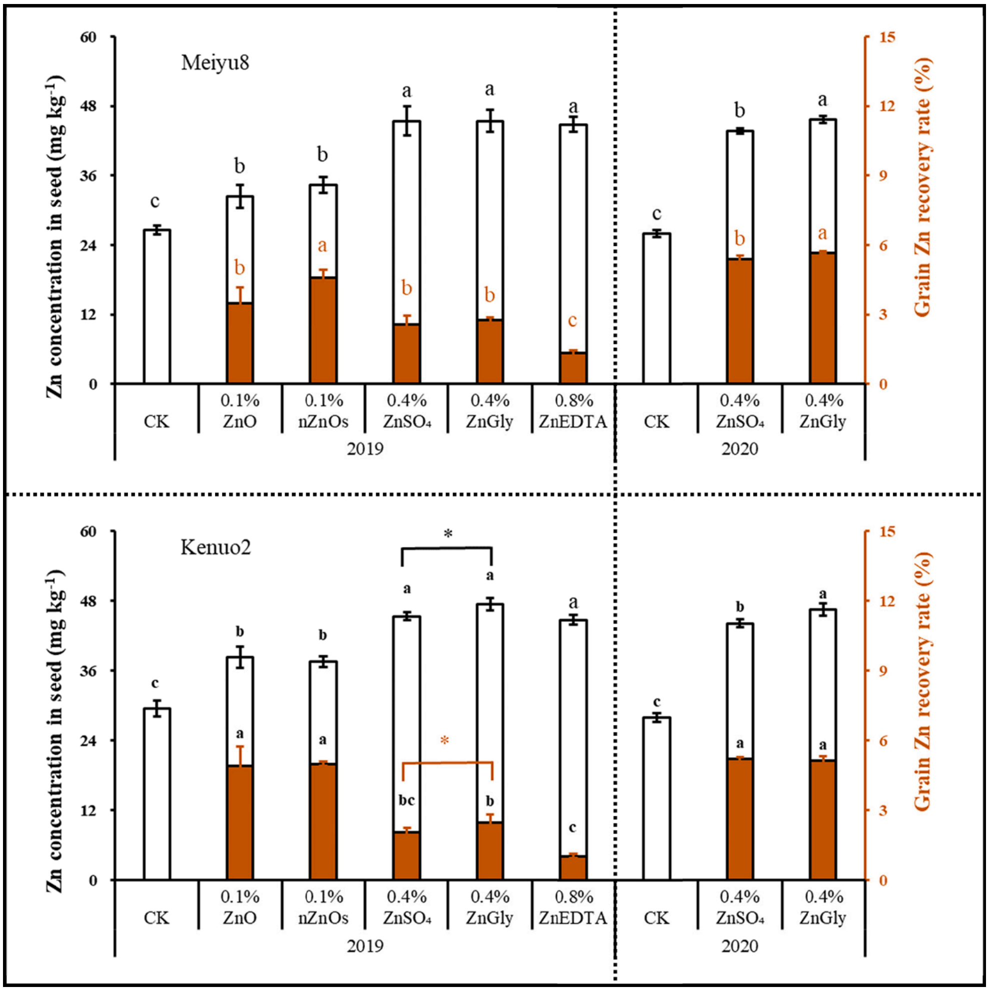

Fig 4. Effects of spraying different Zn formulations on Meiyu8 and Kenuo2 dry seed Zn concentration and grain Zn recovery rates in 2019 and 2020. Different small letters under the same year are significantly different at the 5% significance level. * indicates significant differences ($p < 0.05$). ZnEDTA, ZnGly, and Zn-Gly were set at the same Zn^{2+} concentration gradient as the $ZnSO_4$ treatment.

than from Gly. Furthermore, ZnGly was slightly less mobile in the ear leaves than $ZnSO_4$. Hence, the slow-release rate of ZnGly could reduce localized Zn toxicity, partially inhibit detoxification, and decrease Zn bioavailability.

4.2. Variability in Zn biofortification level

As ZnO and nZnOs had very low aqueous solubility, they limited the improvement in Zn biofortification via foliar fertilization. Moreover, nZnOs had nanoparticle-specific effects on Zn biofortification. In soil applications, ZnEDTA has greater Zn biofortification efficacy than $ZnSO_4$ (Chatterjee & Mandal, 1985; Karak et al., 2005; Zhao et al., 2016). However, the opposite is true for foliar fertilizers (Golden et al., 2016; Wei et al., 2012). Our short-term leaf soaking experiment revealed that $ZnSO_4$ had a lower foliar absorption capacity and mobility than ZnEDTA. Nevertheless, the fertilizer spray applies Zn to the entire plant and not just the leaf tips. ZnEDTA chelate has a relatively high molecular weight and stability constant compared with ZnGly (Dolev et al., 2020; Marešová et al., 2012). Therefore, ZnEDTA chelate may slowly translocate from the leaf to the stalk, the corn cob, and finally, the seeds. Consequently, the ZnEDTA leaf spray yielded the least seed Zn concentrations.

Previous studies have shown that foliar ZnGly achieves higher Zn biofortification than $ZnSO_4$ or ZnEDTA (Ghasemi et al., 2013; Tabesh et al., 2020; Wei et al., 2012). The foliar absorption mechanisms of Gly and ZnGly are poorly understood. The dual-isotope labelling results confirmed that the presence of N in glycine negatively affected Zn absorption in crops, as evidenced by the lower Zn content in the seeds and leaves under ZnGly treatment. The results of the short-term leaf-soaking experiment did not fully reveal why the ZnGly spray maximized seed Zn concentration. However, the labile ZnGly chelate readily released Zn^{2+} at a level of efficacy comparable to that of $ZnSO_4$. A potential reason for the superior ZnGly performance is that it released Zn in the leaves gradually over an extended period.

4.3. Fe:Zn homeostasis in Zn-fortified seed

Foliar Zn spraying simultaneously improves the Zn and Fe concentrations in corn seeds (Wang et al., 2012; Xia et al., 2019). In the present study, among the main elements analyzed, only the standardized Fe:Zn ratio in the Zn-fortified seeds remained relatively steady, regardless of the Zn formulation, spray concentration, or corn cultivar. The underlying mechanism of the synergetic effects involves Zn uptake transporters, such as IRT3 and IRT2 transporters, which can transport both Zn and Fe (Lin et al., 2009). In addition, excess Zn in the leaves and seeds is toxic to plants. The Zn stress can induce the expression of genes related to iron absorption to promote iron absorption, hence alleviating zinc toxicity (Dai, 2021). In global food systems, Zn and Fe deficiencies are closely related; people with Fe deficiency are almost always Zn deficient (Graham et al., 2012; Gregory et al., 2017; Wakeel et al., 2018).

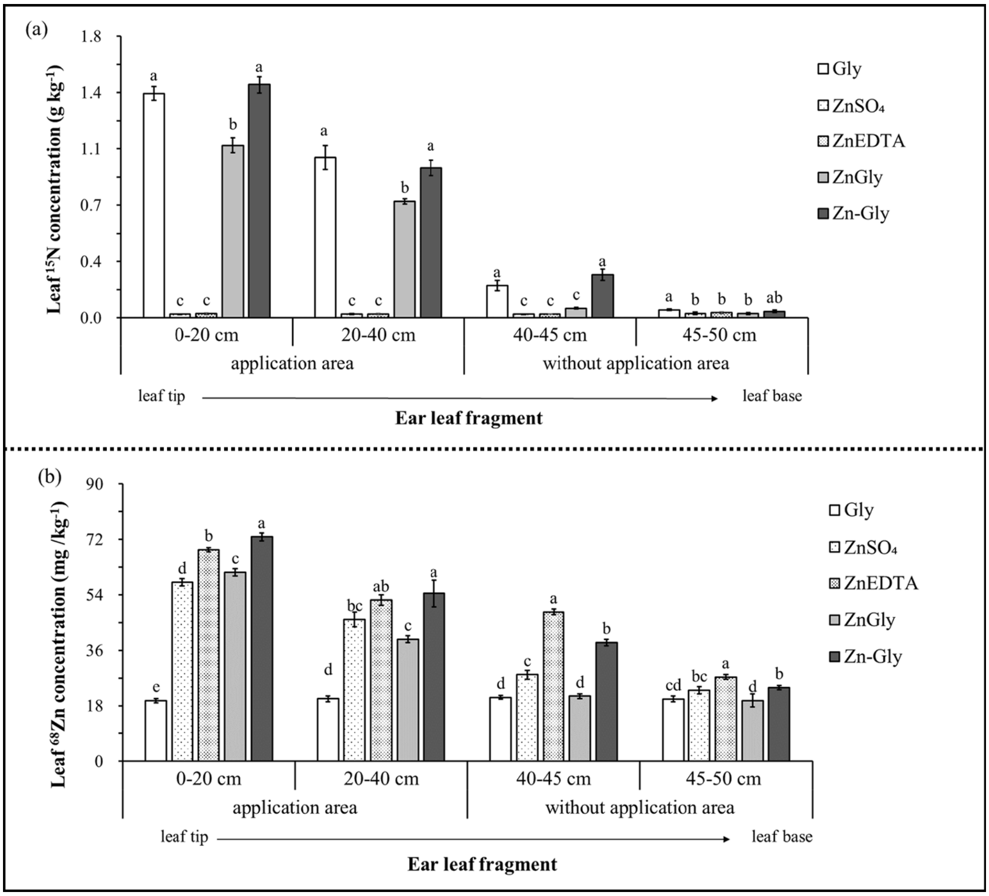

Fig 5. (a) ^{68}Zn and (b) ^{15}N concentrations in the ear leaf sections. Different small letters under the same leaf fragment are significantly different at the 5% significance level.

Therefore, the Fe:Zn homeostasis in seeds has practical significance for human health as certain individuals may be deficient in both Zn and Fe.

4.4. ZnUE and grain Zn recovery rate

Whole-shoot ZnUE is used to evaluate crop fertilizer Zn utilization (Moreira et al., 2018; Wang et al., 2012). However, these parameters do not take into account that only Zn in edible parts is available to consumers; excess Zn accumulation in non-edible parts after foliar Zn application could be phytotoxic to the crops. Wang et al. (2012) used the grain Zn recovery rate to evaluate ZnUE and Zn loss after biofortification. This parameter (i.e., grain Zn recovery rate) is relatively accurate compared with other corn ZnUE parameter. Here, we observed an inverse relationship between grain Zn recovery rate and Zn spraying concentration.

The stable isotope technique, usually ^{15}N tracer, has been applied to accurately calculate *N*-use efficiency (NUE) (Serret et al., 2008; Wallace et al., 2020). Similarly, field-scale studies are required to determine ZnUE. To the best of our knowledge, the present study is the first to use ^{68}Zn to evaluate field-level ZnUE. However, there was a wide gap between the grain Zn recovery rate and the ^{68}Zn recovery rate, with the former overestimating ZnUE. Zn transfer into the seed occurred from not only Zn fertilizer applied to the leaves but also soil and other plant parts. The Zn isotope fractionation effect (Wiggenhauser et al., 2018) and high background ^{68}Zn in seeds could also influence the ZnUE gap. The latter may be ruled out using ^{70}Zn because the isotope has a very low natural abundance. However, it is difficult to eliminate the isotope fractionation effect. Hence, we propose that the "grain Zn recovery rate" first reported by Wang et al. (2012) should be renamed "grain Zn apparent recovery rate" to avoid potential misinterpretation.

5. Conclusions

Without compromising agronomic performance, grain Zn concentration was positively associated with the threshold concentration of Zn sources. For agrochemists, this provides novel insights that could facilitate the development of new Zn fertilizers with high threshold concentrations. Simply adding Gly to $ZnSO_4$ solutions without a chelation process caused more foliar burn than $ZnSO_4$. Notably, both chelated Zn sources have lower phytotoxicity symptoms than $ZnSO_4$. ZnEDTA can be applied up to a concentration of 0.8%, which is higher than the safety threshold concentration (up to 0.4%) of other Zn sources tested. The findings fill a knowledge gap regarding chelated Zn fertilizers, which lowered excess $ZnSO_4$-induced foliar phytotoxicity and exhibited a broader spraying concentration than $ZnSO_4$. However, ZnEDTA had relatively lower biofortification effects than ZnGly and entailed potential environmental risk. ZnGly facilitated the most efficient storage of Zn in the seeds and improved zinc use efficiency. Additionally, glycine can be used as a source of organic N for plants; it is non-toxic to living organisms, making glycine-chelated fertilizers highly suitable for sustainable agriculture. Although the dual-isotope labeling tests confirmed that the presence of N in glycine negatively affected Zn absorption by leaves, the physiological mechanisms of ZnGly with low foliar phytotoxicity and enhancing Zn biofortification of crops require further research to facilitate their extensive application in practice.

CRediT authorship contribution statement

Meng Xu: Investigation, Data curation, Formal analysis, Writing – original draft. **Longgang Du:** Methodology, Investigation. **Mengjiao Liu:** Investigation, Writing – original draft. **Jingjie Zhou:** Visualization.

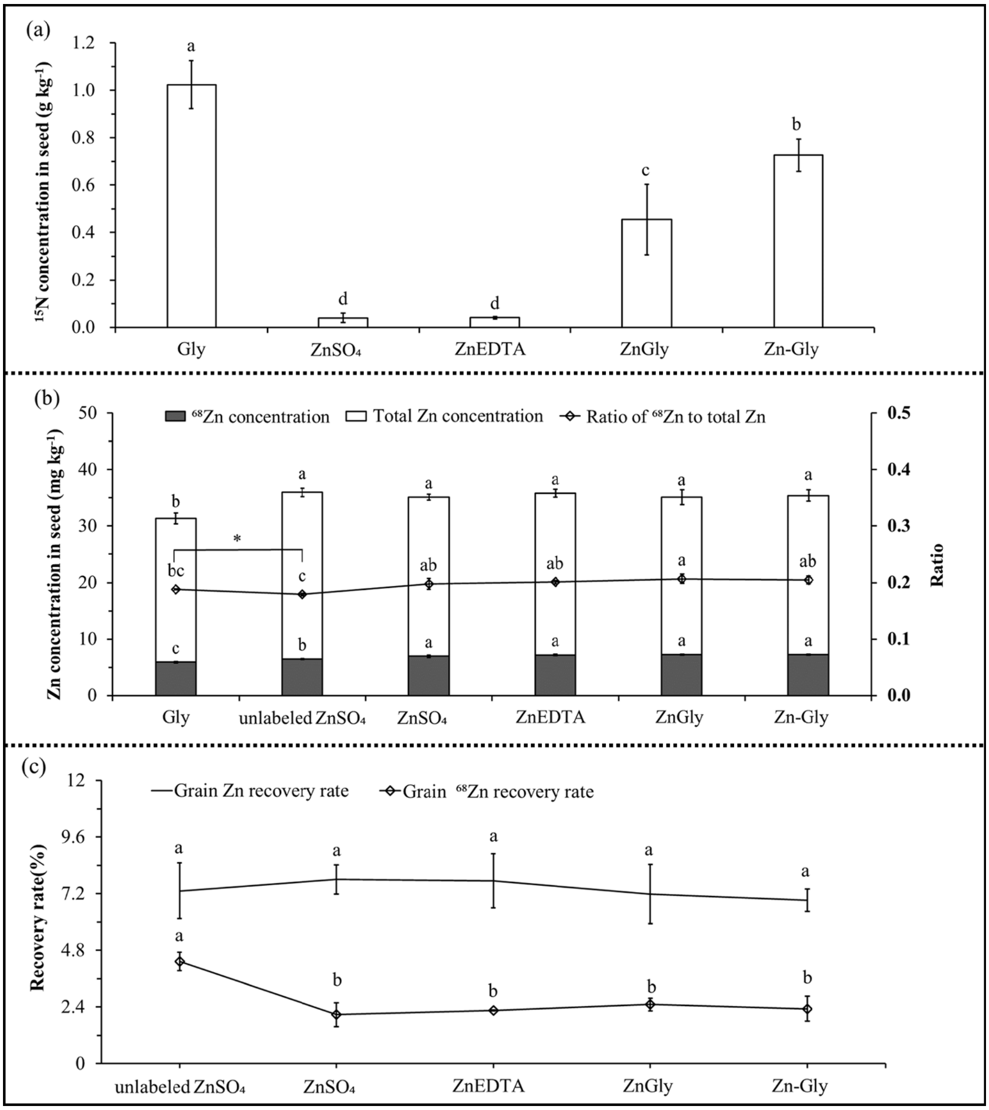

Fig 6. (a) ^{68}Zn and (b) ^{15}N concentrations in the seeds; (c) grain recovery rates of total Zn and ^{68}Zn. Different small letters under the same index are significantly different at the 5% level.

Wankun Pan: Formal analysis. **Haoran Fu:** Methodology. **Xin Zhang:** Software. **Qingxu Ma:** Writing - review & editing. **Lianghuan Wu:** Project administration, Funding acquisition, Supervision, Validation.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2021.131031.

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