Zinc–nitrogen interaction effect on wheat biofortification and nutrient use efficiency

Mónica Montoya1*, Antonio Vallejo1, Jaime Recio1, Guillermo Guardia1, and Jose Manuel Alvarez1

1 ETSI Agronómica, Alimentaria y de Biosistemas, Centro de Estudios e Investigación para la Gestión de Riesgos Agrarios y Medioambientales (CEIGRAM), Universidad Politécnica de Madrid, Ciudad Universitaria s/n, 28040 Madrid, Spain

Abstract

Background: The enhancement of zinc (Zn) concentration in cereal crops without compromising yield is a global challenge with crucial health and food security implications.

Aims: To achieve Zn biofortification in wheat through the appropriate management of fertilization with both Zn and nitrogen (N), due to the synergistic effect between them, using natural organic sources of Zn.

Methods: We carried out a field experiment using a rainfed winter wheat (Triticum aestivum L.) crop fertilized with four Zn sources (Zn-sulphate, Zn-lignosulphonate, Zn-amino acids and Zn-gluconate) and three N application rates under semi-arid conditions.

Results: The strategy of increasing the N rate by 50% with respect to the recommended N rate (i.e., 120 kg N ha−1) did not improve either wheat yield or grain Zn-N concentration. The combined effect of applying natural organic Zn complexes and the recommended N rate tended to increase grain Zn concentrations (by an average of 14%), although this increase was significantly higher when Zn-sulphate was applied (63%) due to its higher recommended Zn application rate. Natural organic Zn fertilizers achieved the highest grain yields, probably due to the enhancement of N uptake. The natural organic Zn fertilizers resulted in higher Zn utilization efficiency compared with the Zn-sulphate fertilizer.

Conclusions: In calcareous Zn-deficient soils, our results suggest that Zn–N co-fertilization involving Zn-sulphate combined with the recommended N application rate would be advisable for obtaining grain Zn biofortification, while the highest yields can be obtained with the application of natural organic Zn fertilizers.

Key words: calcareous soil / foliar–soil application / plant nutrition / soil Zn status / translocation / Zn availability

Accepted December 21, 2019

1 Introduction

Zinc (Zn) is an essential trace element in the nutritional requirements of crop plants, animals, and humans (Hambidge, 2000; Brown et al., 2004; Rehman et al., 2018a). Zinc deficiency in the human diet is an issue of concern in many areas of the world (Hotz and Brown, 2004; Stein, 2010), especially where the diet is cereal-based. In the case of bread-making wheat (Triticum aestivum L.), grain Zn content commonly ranges between 20–35 mg kg−1, which is too low for human daily requirements (Pfeiffer and McClafferty, 2007). Therefore, the enhancement of Zn concentrations to 40–60 mg kg−1 (Graham et al., 2007) in wheat through agricultural practices (biofortification) (Rehman et al., 2018b), is an important global challenge. Such an enhancement would not only improve the nutritional Zn status in millions of people but also crop tolerance to abiotic stresses (Faran et al., 2019).

It is known that the application of Zn fertilization, both foliar and directly to soil, normally increases grain Zn concentration, although its relative efficiency is highly dependent on the source (zinc sulphate, ZnSO4, synthetic chelates or natural complexes) and on soil properties (e.g., pH or texture) (Kumar et al., 2016). Metal-chelating agents play an important role in transporting Zn from solid phases in soils to the surface of plant roots, normally increasing the relative agronomic effectiveness of the source applied to a given soil (Alvarez et al., 2010). However, these synthetic chelates increase farming costs in comparison with mineral sources (Martín-Ortiz et al., 2009). This higher cost is an important barrier to the introduction of these compounds as Zn fertilizers in traditional agriculture.

An interesting possibility for Zn biofortification is the use of natural organic complexes, which are generally cheaper than synthetic chelates. Although their availability in soil is lower than that of synthetic chelates, it is higher than that of ZnSO4 (Goos et al., 2000). There are different commercials products based on lignosulphonates, fulvic and humic acids, gluconates, or even amino acids linked to Zn (Kabata-Pendias and
Montoya, Vallejo, Recio, Guardia, Alvarez

J. Plant Nutr. Soil Sci. 2020, 000, 1–11

Mukherjee, 2007; Lucena et al., 2010) that can be applied as foliar Zn fertilizers. Although it is well documented that Zn application through foliar fertilization produces a Zn mobilization by the phloem and translocation into developing grains in wheat (Erenoglu et al., 2011; Cakmak and Kutman, 2018), to date there is a lack of field experiments evaluating the effectiveness of these natural complexes on Zn biofortification through foliar application. Indeed, most of the information about their effectiveness comes from lab or greenhouse experiments, which can differ from field conditions in terms of soil moisture, root-space limitations, biomass and yield (Alloway, 2008). To our knowledge, no available information exists on the effect of foliar–soil application of natural organic Zn complexes on soil Zn status and Zn uptake by winter wheat under field conditions in calcareous soils.

Recently, N management has also been considered a key agronomic practice to improve Zn biofortification. Some authors have reported that grain Zn concentrations can be enhanced by increasing nitrogen (N) supply, due to a synergistic effect observed in wheat (Kutman et al., 2010; Shi et al., 2010). The absorption and mobility of Zn from the rhizosphere to the grain is dependent on different proteins and other N compounds, including amino acids and peptides (Palmer and Guerinot, 2009). When Zn availability in soil was high or sufficient due to Zn foliar application to the crop, a positive effect of N was observed on Zn biofortification (Gonzalez et al., 2019). A link has also been reported between the remobilization of N and Zn from senescing leaf tissues into wheat grain (Waters et al., 2009). It is also well documented that Zn significantly affects biosynthesis and the structural and functional integrity of proteins (Cakmak et al., 2000; Broadley et al., 2007). Other authors have reported that a high soil N concentration increases biomass yield, reducing Zn grain concentrations, probably due to a secondary “dilution” of this element in the higher biomass (Kabata-Pendias and Pendias, 2001; Miner et al., 2018). This was observed especially for soils with low Zn availability (Zhao et al., 2015). Hence, optimization of Zn also requires optimized N fertilization to ensure the best possible synergistic effect between Zn and N (Cakmak and Kutman, 2018) for each source of Zn. Furthermore, in the case of Zn-deficient soils, wheat grain yields are more sensitive to Zn fertilization than straw yields (Kutman et al., 2010); this is probably due to a Zn-deficiency-induced imbalance in the development of reproductive organs (Cakmak and Engels, 1999). The co-fertilization of Zn with N is, therefore, a recommended agronomic practice to increase grain yield. However, there is no available information about the influence of co-fertilization with N on the effectiveness of natural organic Zn complexes, for improving Zn biofortification or grain yields. The objective of this field experiment was, therefore, to assess the effects of applying different N rates in combination with natural organic Zn sources on Zn biofortification, nutritional quality and yield of a wheat crop. The effects of applying organic and inorganic sources of Zn were also compared. We hypothesized that (1) the foliar application of organic natural Zn sources (lignosulphonates, gluconates or amino acids) to wheat under calcareous soil conditions could achieve a similar or greater efficiency of Zn biofortification than the foliar application of Zn-sulphate, using a significantly lower amount of Zn, and (2) the efficiency of each Zn source would also be dependent on N rate.

2 Material and methods

2.1 Field location and soil characterization

The field experiment was carried out at the National Center of Irrigation Technology, “CENTER” (latitude 40°25′1.31″ N, longitude 3°29′45.07″ W) in Madrid (Spain). Soil texture was silt loam and this soil was classified as a Typic Xerofluvent (Soil Survey Staff, 2014). The main soil characteristics of the topsoil (0–20 cm) were: organic matter (Walkley–Black), 21 g kg⁻¹; total N (Kjeldahl digestion), 1.6 g kg⁻¹; bulk density, 1.3 g cm⁻³; pH_water 8.2; CaCO₃ 82 g kg⁻¹. At the beginning of the experimental period, the NO₃⁻ content was 27 mg kg⁻¹, extractable phosphorous (Olsen) was 29 mg kg⁻¹, DTPA-extractable copper (Cu) was 1.6 mg kg⁻¹, DTPA-extractable Zn was 0.8 mg kg⁻¹, DTPA-extractable manganese (Mn) was 4.6 mg kg⁻¹, and DTPA-extractable iron (Fe) was 4.4 mg kg⁻¹. The chemical analytical procedures used are described in Sparks (1996) and Klute (1986). This native soil is slightly deficient in Zn, with DTPA-Zn < 1 mg kg⁻¹ (Brennan et al., 1993).

2.2 Experimental design

Winter wheat (Triticum aestivum L. var. ‘Ingenio’) was sown on October 27, 2015, at a density of 200 kg seeds ha⁻¹. A total of 60 (5m × 4m) plots were selected and arranged in a randomized block design with 15 Zn source-N rate combinations and four replicates per treatment. Three different N application rates: 0 (N0), 120 kg N ha⁻¹ (the recommended rate considering the average yields for rainfed winter wheat in Central Spain and previous soil analyses, RR), and 180 kg N ha⁻¹ (RR+) were combined with five different Zn sources. Three natural organic Zn sources: Zn-lignosulphonate [ZnLS, 7.5% Zn (w/w)], Zn-amino acids [ZnAA, 6.8% Zn (w/w)] and Zn-gluconate [ZnGluc, 12% Zn (w/w)] (Liiran, 2016) were compared with an inorganic Zn source: ZnSO₄ [ZnSul, 35% Zn (w/w)] and with not applying any Zn (Zn0, 0% Zn).

Zinc sources were split into two dressing applications. On each occasion the application rates were 0 kg Zn ha⁻¹ for Zn0 (0 = abbreviation of zero); 0.18 kg Zn ha⁻¹ for ZnLS, ZnAA and ZnGluc, and 5 kg Zn ha⁻¹ for ZnSul (Alloway, 2008; Liiran, 2016). Consequently, the total Zn rates were 10 kg Zn ha⁻¹ for ZnSul and 0.36 kg Zn ha⁻¹ for the other treatments. The Zn fertilizers were applied by hand with a knapsack sprayer (foliar–soil application) at the beginning of the stem elongation stage (Z30; Zadoks et al., 1974) for the first fertilization and at the end of this stage (Z39) for the second fertilization. In the case of N, urea (46% N w/w), provided by EuroChem Agro GmbH, was applied to the soil surface by hand (120 kg N ha⁻¹ at Z30 for the RR and RR+ treatments and 60 kg N ha⁻¹ at Z39, but only for the RR+ treatments).

2.3 Plant and soil samples

Plant samples were taken at three different intervals: (1) at the end of stem elongation (April 18th), (2) after anthesis (May
20th), and (3) at harvest (June 21th). For the anthesis samples, several young leaves (Zn content is usually more stable in young leaves than in old leaves; Brennan et al., 1993) were collected from three plants and conserved at 4°C to prevent degradation. For the analysis, the samples were defrosted and chopped in order to analyze soluble Zn, and chlorophyll and carotenoid content in fresh matter. The soluble Zn content was determined by 2-(N-morpholino)ethanesulfonic acid (1 mM at pH 6.0) after weighing 0.5 g of leaves (Cakmak and Marschner, 1987). The leaves were macerated in a mortar with 10 mL of this reagent for several minutes. Samples were then centrifuged (10,000 rpm for 15 min), filtered, and measured using flame atomic absorption spectrophotometry (FAAS, Perkin-Elmer Analyst 700). The same equipment was used for analyzing the micronutrients. The chlorophyll and carotenoid contents in fresh leaves were determined following a spectrophotometric method and quantified with the use of the equations described in the Official Methods of Analysis (AOAC, 1990) and according to Lichtenhaler and Wellburn (1983), respectively.

Different plant fractions, i.e., root, stem, leaves and grain, were obtained at both the end of stem elongation and at harvest. In this paper, the term “straw” refers to the shoot excluding the grains at harvest. Total Zn content in dry matter of the different plant fractions at both sampling times was extracted with wet acidic digestion in a digester (SPB 50-24 with an SPB digital controller, Perkin-Elmer), using the method and the equipment described in Montoya et al. (2018).

The Dumas combustion method was used to determine the total protein and N content in the different plant fractions at the end of stem elongation and at harvest, according to the Official Methods of Analysis (AOAC, 1984). Total protein content was calculated by multiplying N content by the factor 6.25 for vegetable matter (FAO, 2004).

Soil samples at depths of 0–10 cm were collected before the second fertilization and after harvest. In both cases, the soil was air-dried and sieved at 2 mm. The diethylentriaminepentaaetate-triethanolamine method (DTPA-TEA: DTPA 5 mM + CaCl₂ 10 mM + TEA 0.1 M, adjusted to pH 7) (Lindsay and Norvell, 1978) was used to determine the concentration of micronutrients potentially available for plants.

### 2.4 Quality attributes

Some of the wheat parameters were studied after harvest in accordance with Jan et al. (2013). Grain yield and straw yield (related to grain, stem and leaf weight) were analyzed for 50 plants of two rows in each plot, which were manually threshed and the grain cleaned. The number of tillers was counted in ten plants of each plot and converted into tillers plant⁻¹. The grain spike⁻¹ parameter was calculated by selecting ten spikes and averaging the number of grains per spike. The 1000-grain weight was recorded by counting and weighing one thousand grains with an electronic balance.

Zinc and N uptake were calculated by multiplying yield by the Zn or N concentration for each plant part and then converting to kg ha⁻¹. Finally, the relative efficiency of fertilizers was estimated as the percentage of their use by the crop (Lu et al., 2012). In this case, the wheat Zn utilization efficiency (ZUE) percentage was defined as:

\[
\text{Zn utilization efficiency} = \frac{\text{Zn uptake}_{\text{fertilized}} - \text{Zn uptake}_{\text{Zn0 (control)}}}{\text{Zn applied}} \times 100^\circ
\]

and the N utilization efficiency (NUE) was calculated as:

\[
\text{N utilization efficiency} = \frac{\text{N uptake}_{\text{fertilized}} - \text{N uptake}_{\text{N0 (control)}}}{\text{N applied}} \times 100^\circ
\]

### 2.5 Statistical analysis

Two-way ANOVAs were carried out to analyze the Zn source, N rates and their interactions with Zn and N content (in grain, plant, root and soil), yield, plant pigments, total uptake of Zn and N, ZUE, and NUE. Means were separated using the least significant difference (LSD) test and a probability level of \( p < 0.05 \) was selected to establish statistical significance. Pearson’s correlation coefficients were used to analyse the relationships between total grain Zn and N concentration, grain yield, yield parameters, total Zn and protein in leaves after anthesis, soluble Zn in leaves, total Zn uptake, chlorophyll a and total Zn-DTPA in soil. All statistical analyses were performed using Statgraphics Centurion version XVI software (Manugistic Inc., Rockville, MD, USA).

### 3 Results

#### 3.1 Yields at harvest

All Zn sources numerically increased grain yields, but these increases were only significant for the organic sources (Tab. 1). The application of 120 kg N ha⁻¹, which was the recommended N rate (RR treatments) for the region, increased grain yield by 13% with respect to the N0 treatment. By contrast, increasing the N rate to 180 kg N ha⁻¹ (RR+ treatments) did not improve the wheat yield in comparison with the N0 treatment. The number of grains per spike increased in the following order: N0 < RR+ < RR (\( p < 0.05 \)), and an enhancement of the number of tillers per plant was also observed in the RR and RR+ treatments, in comparison with N0, for almost all the Zn treatments (including Zn0), except ZnAA (see Tab. S1). Grain yield showed a significant and positive correlation with the number of grains per spike (\( R^2 = 24\% \), \( p < 0.001 \)) and the number of tillers per plant (\( R^2 = 39\% \), \( p < 0.001 \)).

The Zn–N interaction effect was significant for straw and leaf yields (Tab. S1), with the highest values being obtained for the combination of the natural organic Zn sources with the RR treatment. This interaction did not affect stem yield (see Tab. S1), although this property was strongly dependent on N fertilization. In addition, the natural organic Zn treatments produced a significant increase in stem yield compared to the Zn0 and ZnSul treatments (see Tab. 1).
The increase in grain Zn concentration in comparison with N0Zn0 was very dependent on the Zn–N interaction, ranging from 5.8 mg Zn kg⁻¹ (for the N0ZnGluc treatment) to 28.6 mg Zn kg⁻¹ (for the RRZnSul treatment) (Tab. 2). For the natural organic Zn sources, a numerically greater increase in the Zn concentration was observed with N fertilization (N0 < RR < RR+), with the highest Zn concentration observed for the RR+ZnGluc treatment (49.8 mg Zn kg⁻¹ grain). For the inorganic Zn source, the RRZnSul treatment gave the highest Zn concentration (63 mg Zn kg⁻¹ grain). The grain Zn concentration generally increased (p < 0.05) in the Zn-fertilized treatments. This effect was significant for all N0 treatments but not for all the RR and RR+ treatments.

Regarding grain N concentrations, the addition of N or Zn fertilizers had a positive effect. Grain N concentration increased in the order N0 < RR < RR+. Significant increments in grain N concentration in Zn-fertilized plots with respect to N0Zn0 were only observed for N0ZnAA and N0ZnGluc. Total Zn concentration in grain was positively correlated to total N concentration (R² = 30%, p < 0.001).

The micronutrient (Cu, Mn, and Fe) grain concentrations ranged from 5 to 7 mg Cu kg⁻¹, 25 to 31 mg Mn kg⁻¹, and 30 to 35 mg Fe kg⁻¹. For the grain Cu concentration there were no significant differences between treatments, whereas both N application rates were associated with decreases in grain Mn concentrations in comparison with the N unfertilized treatment (data not shown). The Zn–N interaction had a significant effect on the grain Fe concentration (Tab. 2), with the highest values being associated with the combination of RR+ and Zn fertilizers (independent of the Zn source).

### 3.3 Zn and N in vegetal tissues

The application of Zn fertilizers enhanced the concentration of both total and soluble Zn in leaves, although the ZnSul treatment had significantly higher concentrations than the other Zn treatments, regardless of the date and N application rate (Tab. 3). Soluble Zn was positively correlated with total Zn in leaves after anthesis (R² = 44%, p < 0.001) and total Zn concentration in the stems, by 2 to 10 mg Zn kg⁻¹ (in comparison with the Zn0 treatment). The use of N fertilization also enhanced the total stem Zn concentration, but no differences between N rates (RR and RR+) or significant Zn–N interaction effects were observed (Tabs. 3 and S2).

Total protein concentration in leaves was reduced by 73% on average at harvest compared with after anthesis. Total protein in leaves after anthesis had a significant positive correlation with total grain N concentration (R² = 42%, p < 0.001).

### 3.4 Indicators of plant nutrition

Plant pigment content (chlorophyll and carotenoids), in leaves are presented in Tabs. S3 and S4. Grain yield and grain N content were positively correlated with chlorophyll a (R² = 13%, p < 0.01 and R² = 49%, p < 0.001, respectively).
Regardless of the Zn source, Zn fertilization increased all of these parameters, with higher values obtained for the natural organic sources than for the inorganic source.

### Table 2: Total Zn, N and Fe concentrations in grain for the different Zn sources (control without Zn, Zn0; Zn-sulphate, ZnSul; Zn-lignosulphonate, ZnLS; Zn-amino acids, ZnAA; Zn-gluconate, ZnGluc) and three N application rates (0 kg N ha$^{-1}$; N0, 120 kg N ha$^{-1}$, RR; 180 kg N ha$^{-1}$, RR+).$

<table>
<thead>
<tr>
<th>Effect</th>
<th>Total Zn conc. grain (mg kg$^{-1}$)</th>
<th>Total N conc. grain (%)</th>
<th>Total Fe conc. grain (mg kg$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Interaction zinc × nitrogen</td>
<td>**</td>
<td>*</td>
<td>**</td>
</tr>
<tr>
<td>N0Zn0</td>
<td>34.40 B b</td>
<td>1.90 B b</td>
<td>30.34 B b</td>
</tr>
<tr>
<td>RRZn0</td>
<td>38.81 B c</td>
<td>2.73 A a</td>
<td>32.70 AB a</td>
</tr>
<tr>
<td>RR+Zn0</td>
<td>45.23 A b</td>
<td>2.94 A a</td>
<td>33.15 A a</td>
</tr>
<tr>
<td>N0ZnSul</td>
<td>41.27 B a</td>
<td>1.97 B b</td>
<td>29.24 B b</td>
</tr>
<tr>
<td>RRZnSul</td>
<td>63.06 A a</td>
<td>2.96 A a</td>
<td>34.5 A a</td>
</tr>
<tr>
<td>RR+ZnSul</td>
<td>58.96 A a</td>
<td>3.03 A a</td>
<td>34.83 A</td>
</tr>
<tr>
<td>N0ZnLS</td>
<td>40.80 A a</td>
<td>1.96 B b</td>
<td>29.25 B c</td>
</tr>
<tr>
<td>RRZnLS</td>
<td>43.25 A bc</td>
<td>2.98 A a</td>
<td>32.36 AB b</td>
</tr>
<tr>
<td>RR+ZnLS</td>
<td>44.40 A b</td>
<td>3.11 A a</td>
<td>35.09 A a</td>
</tr>
<tr>
<td>N0ZnAA</td>
<td>41.37 B a</td>
<td>2.34 B a</td>
<td>33.28 A a</td>
</tr>
<tr>
<td>RRZnAA</td>
<td>43.31 AB bc</td>
<td>2.84 A a</td>
<td>32.02 A a</td>
</tr>
<tr>
<td>RR+ZnAA</td>
<td>47.62 A b</td>
<td>2.97 A a</td>
<td>33.79 A a</td>
</tr>
<tr>
<td>N0ZnGlu</td>
<td>40.24 B a</td>
<td>2.44 B a</td>
<td>30.98 B b</td>
</tr>
<tr>
<td>RRZnGlu</td>
<td>46.70 A b</td>
<td>2.90 A a</td>
<td>31.63 B b</td>
</tr>
<tr>
<td>RR+ZnGlu</td>
<td>49.81 A b</td>
<td>3.06 A a</td>
<td>34.88 A a</td>
</tr>
<tr>
<td>S.E.</td>
<td>1.87</td>
<td>0.09</td>
<td>0.76</td>
</tr>
</tbody>
</table>

**Nitrogen**

| N0            | 39.62 c | 2.12 c | 30.62 c |
| RR            | 47.03 b | 2.88 b | 32.64 b |
| RR+           | 49.20 a | 3.02 a | 34.35 a |
| S.E.          | 0.84    | 0.04   | 0.34    |

**Zinc**

| Zn0           | 39.48 c | 2.53 b | 32.06 |
| ZnSul         | 54.43 a | 2.66 ab | 32.86 |
| ZnLS          | 42.82 b | 2.68 a | 32.23 |
| ZnAA          | 44.10 b | 2.71 a | 33.03 |
| ZnGluc        | 45.58 b | 2.79 a | 32.50 |
| S.E.          | 1.08    | 0.05   | 0.44   |

*Different letters within columns indicate significant differences by applying the LSD test at $p < 0.05$. Standard error (SE) is given for each effect. *, ** and *** denote significant at $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively. "NS" denotes not significant. Different capital letters in the interaction indicate significant differences between N rates within a Zn treatment. Different lowercase letters indicate significant differences between Zn treatments within the same N rate, by applying the LSD test at $p < 0.05$.

---

### 3.5 Zn and N in roots

The Zn concentration in roots (Fig. S1a) was enhanced by a factor of 1.02–3.06 in the Zn-fertilized treatments in comparison with the Zn0 treatment, with small differences between different Zn sources. There was, however, a significant increase with the ZnSul treatment for both sampling periods;
this suggests that an important part of the Zn applied was finally released to the soil. The RRZnSul treatment gave the highest root Zn concentration values.

Zn fertilization generally increased the N root concentration with respect to Zn0 (Fig. S1b). For the different N application rates, N contents in roots in RR were significantly higher than those of N0, particularly for the first fertilization, but no significant differences were observed with respect to the RR+ treatments.

### 3.6 Total Zn and N uptake

The use of Zn fertilizers (both inorganic and natural organic sources) significantly increased Zn uptake in the whole plant (grain, stem, and leaf) (Fig. S2). In addition, N application improved total Zn uptake even in the treatment without Zn supply.

Total N uptake increased significantly throughout the whole plant in the N-fertilized treatments in comparison with the N0 treatments. Even so, no significant differences were observed between the two N application rates. The application of an organic source of Zn effectively increased N uptake in non-N fertilized soils (in NOZnAA and NOZnGluc, it was 127.2 kg N ha⁻¹ and 132.7 kg N ha⁻¹, respectively), reaching similar values to those obtained by the corresponding RR treatment (152.6 kg N ha⁻¹).

### 3.7 Zn and N utilization efficiency

Regardless of the Zn source, the RR treatments produced the highest total Zn and N utilization efficiency (Fig. 1). By contrast, the highest N rate decreased both Zn and N utilization efficiency for all Zn sources. The most efficient treatments with regards to Zn utilization (Fig. 1a) were the combination of RR and the natural organic Zn fertilizers. The Zn–N treatments had a NUE greater than 40%, except the RR+ZnAA and RR+ZnGluc treatments (Fig. 1b).

### 3.8 Zn availability in the soil

Zn fertilizers were applied by the foliar–soil method and so a high proportion of the sprayed fertilizer was deposited in the soil, especially for the first fertilization when there was less canopy. The fertilizers with Zn, independently of the Zn source and the N application rates, numerically increased Zn availability in the soil above the Zn deficiency threshold (≤ 1 mg Zn kg⁻¹ soil) (Fig. 2). The Zn availability in the soil was affected by the N rate in the case of the inorganic fertilizer (in the first fertilization), but not when natural organic Zn sources were used. The ZnSul treatment produced the highest Zn availability regardless of the N application (p < 0.05).

Total Zn-DTPA in the soil after the first fertilization and at harvest was positively correlated to total Zn uptake (R² = 69%, p < 0.001 and R² = 58%, p < 0.001, respectively) and total

<table>
<thead>
<tr>
<th>Effect</th>
<th>Total Zn conc. DM stem</th>
<th>Total Zn conc. DM leaves after anthesis</th>
<th>Total Zn conc. DM leaves after harvest</th>
<th>Soluble Zn conc. DM leaves after anthesis</th>
<th>Total protein conc. DM stem</th>
<th>Total protein conc. DM leaves after anthesis</th>
<th>Total protein conc. DM leaves after harvest</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nitrogen</td>
<td>***</td>
<td>***</td>
<td>***</td>
<td>***</td>
<td>***</td>
<td>***</td>
<td>***</td>
</tr>
<tr>
<td>N0</td>
<td>6.23 b</td>
<td>17.18 b</td>
<td>23.05 b</td>
<td>15.68 b</td>
<td>3.07 c</td>
<td>23.07 c</td>
<td>5.14 b</td>
</tr>
<tr>
<td>RR</td>
<td>11.55 a</td>
<td>21.32 a</td>
<td>28.96 a</td>
<td>20.31 a</td>
<td>5.38 b</td>
<td>27.54 b</td>
<td>8.35 a</td>
</tr>
<tr>
<td>RR+</td>
<td>12.33 a</td>
<td>19.54 a</td>
<td>24.51 a</td>
<td>18.31 a</td>
<td>6.21 a</td>
<td>28.58 a</td>
<td>8.19 a</td>
</tr>
<tr>
<td>S.E.</td>
<td>0.69</td>
<td>0.96</td>
<td>3.09</td>
<td>1.08</td>
<td>0.24</td>
<td>0.34</td>
<td>0.27</td>
</tr>
<tr>
<td>Zinc</td>
<td>***</td>
<td>***</td>
<td>***</td>
<td>***</td>
<td>*</td>
<td>**</td>
<td>**</td>
</tr>
<tr>
<td>Zn0</td>
<td>6.37 c</td>
<td>15.64 c</td>
<td>10.74 c</td>
<td>14.44 c</td>
<td>4.29 b</td>
<td>26.01 bc</td>
<td>6.18 c</td>
</tr>
<tr>
<td>ZnSul</td>
<td>16.50 a</td>
<td>30.42 a</td>
<td>71.65 a</td>
<td>24.17 a</td>
<td>4.86 ab</td>
<td>25.11 c</td>
<td>7.16 a–c</td>
</tr>
<tr>
<td>ZnLS</td>
<td>8.52 b</td>
<td>15.47 c</td>
<td>14.50 b</td>
<td>17.38 b</td>
<td>4.68 b</td>
<td>27.35 a</td>
<td>7.10 bc</td>
</tr>
<tr>
<td>ZnAA</td>
<td>9.15 b</td>
<td>16.93 bc</td>
<td>14.82 b</td>
<td>17.60 b</td>
<td>4.90 ab</td>
<td>26.95 ab</td>
<td>7.57 ab</td>
</tr>
<tr>
<td>ZnGluc</td>
<td>9.64 b</td>
<td>18.29 b</td>
<td>15.81 b</td>
<td>16.90 b</td>
<td>5.72 a</td>
<td>26.55 ab</td>
<td>8.12 a</td>
</tr>
<tr>
<td>S.E.</td>
<td>0.89</td>
<td>1.24</td>
<td>3.99</td>
<td>1.39</td>
<td>0.31</td>
<td>0.43</td>
<td>0.34</td>
</tr>
</tbody>
</table>

*aDifferent letters within columns indicate significant differences by applying the LSD test at p < 0.05. Standard Error (S.E.) is given for each effect. *, ** and *** denote significant at p < 0.05, p < 0.01, and p < 0.001, respectively. "NS" denotes not significant. DM = dry matter.*

Table 3: Soluble Zn, total Zn and protein in several parts of wheat for the different Zn sources (control without Zn, Zn0; Zn-sulphate, ZnSul; Zn-lignosulphonate, ZnLS; Zn-aminoacids, ZnAA; Zn-gluconate, ZnGluc) and three N application rates (0 kg N ha⁻¹, N0; 120 kg N ha⁻¹, RR; 180 kg N ha⁻¹, RR+).
4 Discussion

4.1 Effect of Zn and N co-fertilization on plant yield and quality

Our results demonstrate that the foliar–soil application of Zn fertilizers could be an effective strategy to increase grain Zn concentrations, without penalizing other micronutrients (Cu, Mn or Fe). However, the efficiency of Zn biofortification was highly dependent on the Zn source and N rate applied. For the cv. Ingenio wheat variety, under our experimental conditions, all Zn sources maintained the grain Zn concentration within the target of 40–60 mg Zn kg⁻¹, considered adequate to meet the Zn requirement for humans (Gawalko et al., 2002). Co-fertilization with N also increased the Zn concentration in each part of the plant (grain, stem, and leaf) in winter wheat. This positive effect of Zn and N fertilization on Zn biofortification that was observed for most of the Zn treatments has also been described in several previous experiments using ZnSO₄ (ZnSul) (e.g., Kutman et al., 2010; Xue et al., 2012; Gonzalez et al., 2019). These authors suggested that grain proteins act as Zn sinks, which could explain this synergistic effect. Indeed, we observed a positive correlation between the total N and Zn concentrations in the grain (see section 3.2). The accumulation of Zn in the grain can occur via three physiological steps: root uptake, root-to-shoot translocation, and the remobilization of Zn (Erenoglu et al., 2011). Consequently, the Zn distribution is constantly changing inside the plant, from the moment of Zn fertilization through to harvest. According to Cakmak et al. (2010), Zn remobilization from vegetative tissues to the grains seems to be an important mechanism for enhancing grain Zn concentration. Waters et al. (2009) and Marschner (2012) reported that 70% of the Zn reserves in the vegetative tissues of wheat were remobilized and that this was particularly evident during the leaf senescence and grain filling stages (Xue et al., 2012). In our study, this effect was not clearly observed and the total Zn concentration in leaves after anthesis was similar to that at harvest. Concurrent Zn uptake during grain-filling has also been proposed as an important source of Zn in grain (Waters and Grusak, 2008). Garnett and Graham (2005) found that most of the Zn allocated to wheat grain was the result of Zn entering shoots after anthesis. In that case, Zn translocation occurred via phloem and through the metal-binding proteins of the sap (Page and Feller, 2005; Page et al., 2006). We consider that this could also have been a plausible source of Zn in our experiment.

The inorganic source (ZnSul), which was applied at the recommended rate of 10 kg Zn ha⁻¹, produced higher stem and leaf Zn concentrations, both after anthesis and after harvest, in comparison with natural organic Zn sources (with Zn being applied at a commercially recommended rate of 0.36 kg Zn ha⁻¹) (see section 3.3). The high Zn rate used for the inorganic treatment explains these higher Zn concentrations in vegetal tissues, and particularly, in the soil after anthesis.

The results of Zn concentrations in grain for the RR+ treatments only surpassed those of the RR treatments when no Zn was applied (Tab. 2). RR was therefore considered the best option for reducing economic and environmental costs. Our results (i.e.,

![Figure 1: Total Zn (a) and N (b) utilization efficiency for different parts of wheat (grain, stem and leaves) during the sampling period for the different Zn sources (control without Zn, Zn0; Zn-sulphate, ZnSul; Zn-lignosulphonate, ZnLS; Zn-amino acids, ZnAA; Zn-glucenate, ZnGluc) and three N application rates (0 kg N ha⁻¹, N0, 120 kg N ha⁻¹, RR, 180 kg N ha⁻¹, RR+). Statistical differences at p ≤ 0.05 (LSD test) are presented by different lowercase letters for each plant part and different capital letters for the whole plant. Non-significant differences are presented without letters. The vertical line in each column represents the standard deviation from the mean of each treatment for the whole plant.](image-url)
similar Zn biofortification rates for RR and RR+ concurred with those of Wang et al. (2018), who reported that increasing N fertilization had no negative consequences for Zn enrichment in grain. On the contrary, Lu et al. (2010) and Zhao et al. (2015) observed that treatments with the application of Zn and high dose of N often produced a secondary “dilution” effect (i.e., a reduction in the Zn concentration in grain associated with high N doses). However, this dilution effect for grain Zn may only occur under different climatic conditions, e.g., higher-yielding wheat and higher N uptake.

In our study, the use of natural organic Zn fertilizers enhanced the chlorophyll a content more than the inorganic Zn fertilizer. The latter affected photosynthesis and also resulted in higher biomass and grain yields (Alloway, 2008; Rehman et al., 2019) (see Tabs. S3 and 2). To our knowledge, there is no available information about the effects of natural organic Zn complexes on wheat grain yield under field conditions in comparison with the application of ZnSul. The increase in grain yield associated with these treatments was in line with the increase in the number of tillers and grains per spike. For rice, Rana and Kashif (2014) reported that the ZnSul source yielded fewer tillers per plant than other Zn sources. Although other authors have suggested that the numbers of tillers per plant can be increased by enhancing the soil Zn concentration and foliar–soil Zn application (Jan et al., 2013; Shivay et al., 2015), the highest soil Zn concentration in our experiment was obtained with the ZnSul treatments, and no significant effect was observed regarding the number of tillers. Furthermore, in the treatments in which no N was applied, the natural organic Zn fertilizers were associated with higher N uptake values (approximately 120 kg N ha⁻¹ on average) than the inorganic Zn source (78 kg N ha⁻¹). More studies should therefore be carried out in order to clarify why the Zn source is apparently key for N uptake and for yield components.

On the other hand, our results show that natural organic Zn sources (without any N application) applied as foliar fertilizers produced a similar level of N enrichment in grain and straw to the application of 120 kg N ha⁻¹ without Zn fertilization. Future studies should confirm whether it is possible to reduce the N rate to below the recommended rate by using Zn fertilizers, while still maintaining grain and straw quality. This hypothesis is compatible with Venterea et al. (2016) and Cakmak et al. (2017), who suggested that a suitable nutrient balance requires an appropriate fertilization management of macronutrients and their interactions with micronutrients.

An additional positive effect of using N fertilizers was the Fe biofortification in the grain. These results were in line with those reported by Almendros et al. (2019), who suggested that the application of N in the form of urea or pig manure resulted in an increase in barley grain Fe concentrations. The transfer of the Fe cation from the rhizosphere to the grain is dependent on several different proteins and could be enhanced by N fertilization (Palmer and Guerinot, 2009). Grain protein is also a sink for Fe and, therefore, N fertilization would favor the deposition of this element in the grain (Zhao et al., 2009; Aciksoz et al., 2011a).

4.2 Effect of Zn and N co-fertilization on soil Zn status

Liu et al. (2016) reported that high-yielding wheat crops needed a concentration of 2 mg Zn DTPA-extractable kg⁻¹ soil at crop maturity through a soil application strategy. Those findings are not consistent with our results, i.e., higher yields in some treatments (e.g., ZnAA or ZnGluc) with a lower concentration of soil DTPA-Zn (< 2 mg kg⁻¹) compared with ZnSul. Our results suggest that a Zn concentration in the soil above the critical deficiency threshold concentration (1 mg Zn...
DTPA-extractable kg^{-1}) is sufficient to increase grain Zn biofortification (see Fig. 2). Although Zn uptake by the root system is the beginning of the Zn route (root-shoot-grain), a soil–foliar fertilization strategy could also increase the Zn concentration through leaf uptake, which would complement the effect of the soil Zn available. As in Liu et al. (2016), the highest grain Zn biofortification rates were obtained with > 4 mg Zn DTPA-extractable kg^{-1} (see Fig. 2).

Regarding the Zn–N interaction in the ZnSul treatment, Prasad (2005) and Neue et al. (1998) reported that the use of N fertilizer combined with ZnSul increased Zn availability and solubility in the soil, which is in agreement with our results at the first fertilization. This synergistic effect between Zn and N (i.e., increase of soil Zn availability and solubility) could be due to rhizosphere-induced acidification, resulting from excess of cations (especially NH4+) over anions and the simultaneous release of H+ from the roots.

On the other hand, Paterson et al. (2006) and Aciksoz et al. (2011b) reported that Zn mobilization and uptake could be affected by an increase in the N supply due to the stimulation of soil microbial activity and increased root exudation. Rehman et al. (2018c) and Rehman et al. (2018d) also reported that root exudation (e.g., in the form of organic acids) increased the solubility and availability of nutrients due to changes in the pH of the rhizosphere and in soil microbial activity. Consequently, this enhanced phytosiderophore production and thus Zn acquisition by the plants. Other authors have suggested that the increase of Zn uptake could also be due to the abundance of root Zn uptake proteins including ZIP family proteins (e.g., IRT1) (Hall and Williams, 2003; Ishimaru et al., 2005).

Under semi-arid conditions the topsoil often has a low moisture content for several days or weeks. This occurred in our experiment during the reproductive growth stage and, hence, root activity generally declined due to the reduction in photo-assimilate allocation (Zhang et al., 2012). Applying foliar or foliar–soil Zn fertilization during the reproductive growth stages, as in this study, is an appropriate field practice for enhancing Zn accumulation (Cakmak et al., 2010). More studies are needed to clarify the N and Zn efficiency under different stress and climatic conditions (e.g., in very wet or very dry years and in humid or dry areas).

The natural organic Zn sources produced higher ZUE values than the inorganic fertilizer (Fig. 1). Even so, this effect was very closely related to the recommended dose for each Zn fertilizer (see section 2.2), as confirmed by Martin-Ortiz et al. (2009) and Alvarez and Gonzalez (2006) for wheat and maize, respectively. The application of natural organic Zn complexes could have enhanced the diffusion flow in this calcareous soil, favoring the Zn uptake by wheat roots at a lower Zn soil concentration and improving Zn utilization efficiency. Although the main disadvantage of the inorganic Zn fertilizer was that it gave a lower grain yield, it has the advantage of a lower cost than natural organic Zn complexes.

### 5 Conclusions

Our results suggest that RRZnSul is the most suitable treatment to obtain greater Zn concentrations, while the RRAA and RRGluc would be the better treatments for obtaining high yields (possibly as a result of the increased number of tillers per plant). Additionally, the use of Zn fertilizers is also advisable for obtaining better quality wheat through the increase of total grain N concentration, in a context of reducing N supply. Foliar-soil Zn fertilization using natural organic Zn fertilizers (ZnLS, ZnAAA, and ZnGluc) can be considered an appropriate fertilization approach due to the high efficiency of these fertilizers in terms of improving Zn uptake in a winter wheat crop and avoiding Zn overload in the soil. This study also demonstrated that the DTPA-TEA extraction method to determine potential Zn availability for plants can be used partially as an indicator for the optimization Zn fertilization with the objective of increasing the grain Zn concentration of winter wheat. To obtain high yields and Zn biofortification (40–60 mg kg^{-1} Zn in grain) in Zn-deficient calcareous soils, the concentration of available Zn in the soil should be > 1 mg kg^{-1} soil, taking into account the Zn uptake by the plant through foliar application.

Our study showed that Zn–N co-application can be a sustainable strategy for enhancing crop quality and yields and, increasing N use efficiency in Zn-deficient soils (e.g., soils with high pH, clay, and carbonate content).

### Acknowledgments

Financial support was provided by an ERDF co-financed grant, AGL2015-64582-C3-3-R (MINECO-FEDER), from the Ministerio de Economía y Competitividad (Spanish Government). We are grateful to the Comunidad de Madrid (Spain) and Structural Funds 2014–2020 (ERDF and ESP) for the financial support (project AGRISOST-CM S2013/ABI-2717). M. Montoya is the recipient of the FPI grant BES-2016-076712. Special thanks are given to the field assistants working with us at CENTER, particularly to A. Sánchez-De Ribera. This work was done within the framework of the Moncloa Campus of International Excellence (UCM-UPM).

### References


