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Zn-DTPA-HEDTA-EDTA application: a strategy to improve the yield and plant quality of a barley crop while reducing the N application rate

Running-title: Zn-DTPA-HEDTA-EDTA application to reducing the N application rate

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Abstract

Over-use of N fertilization has been common in order to obtain the highest possible grain yield. This experiment was conducted to investigate the efficiency of combining the application of N (30, 60 or 90 kg ha^{-1}; in the form of pig slurry –PS- or urea) and ZnCHE (0, 0.5, 1, or 1.5 kg ha^{-1}). Nitrogen fertilization combined with soil Zn applications had a significant interaction on various plant parameters (including grain protein concentration, yield, Zn uptake and N uptake). An application rate of 90 kg N ha^{-1} seems recommendable to obtain high values for both crop yield and N uptake by the plant. PS application was associated with higher mean grain yield and Zn utilization than urea application, but with lower grain protein concentration. On the other hand, the lowest Zn application rate (0.5 mg Zn kg^{-1}) was sufficient to achieve a high grain yield (> 3200 kg ha^{-1}). Higher Zn rates (1 and 1.5 mg Zn kg^{-1}) provided great Zn concentrations in different parts of the plant (grain, leaf, stem and root). Furthermore, high grain protein concentrations (> 9.6%) were obtained with combinations of N60 or N90 and ZnCHE-1 or ZnCHE-1.5, both for PS and for urea. The application of this synthetic Zn chelate could be

recommended as a strategy for reducing the N application rate but still obtaining high grain yield and nutritional value in barley.

Keywords

N-Zn combined fertilization, yield, nutritional composition, protein, agronomic efficiency, utilization efficiency

1. Introduction

Barley (*Hordeum vulgare* L.) is primarily used for both malt and beer production and as animal feed; it is also used as food for human consumption. Barley grain is high in carbohydrates and also contains moderate amounts of protein, calcium and phosphorus and small quantities of vitamin B. Barley is the world's fourth most important cereal crop (after wheat, rice and maize) in terms of both quantity of production and area of cultivation. The increase in the total barley production over the last half-century has mainly been due to increases in yield, since the total area under production has remained relatively stable, or has even decreased in recent years (FAOstat, 2018). Hordeum species are found in most areas with Mediterranean climates.

Nitrogen (N) is an essential element for crop development. The rate of N fertilizer application is critical for cereal crops since N affects grain yield and plant quality (Lopez-Bellido, 2009). Under Mediterranean climatic conditions, barley crops under rainfed Mediterranean conditions extract approximately 25 kg of N per 1000 kg of grain produced from the soil. The need for N fertilization in barley crops differs from region to region. Under wet conditions, such as parts of the north of the Spain, with barley yields of between 3000 and 5300 kg ha^{-1}, it is recommended to apply N at a rate of between 80 and 140 kg N ha^{-1}. In contrast, in the arid drylands of Castilla-La Mancha, with average barley yields of around 2000 kg ha^{-1}, the N application rate tends to be below 50 kg N ha^{-1}. In irrigated areas, with yield levels above 5000 kg ha^{-1}, the typical N application rate is around 125 kg N ha^{-1} (Lopez-Bellido, 2009).

While the need for greater food production has increased, the global consumption of N in its synthetic (commercial) and organic (manure) forms has increased at an even greater rate. Several organic and inorganic N sources can supply the N required for optimum crop growth. In 2018, Spain was the largest pig producer in the EU (Eurostat, 2018). These animals produce large quantities of manure every year, which presents a problem in terms of the management of

the very large volume of waste produced. The agricultural use of this manure is recommended not only for fertilizing but also to facilitate the disposal of these increasingly important residues. Given its value as a fertilizer, pig slurry is commonly used in agriculture and this is a relatively cheap way of decomposing it and offers a low-cost alternative to mineral fertilizers. However excessive applications of manure may cause the pollution of groundwaters as a result of leaching, especially involving nitrates.

In various regions of Spain, it has been traditional to apply high doses of N fertilizer with the sole aim of achieving high yields. However, there are several drawbacks associated with the over-use of N fertilizers: 1) the cost; from an agronomical point of view over-use does not produce a significant increase in crop yield; and 2) environmental problems; normally associated with N losses to the natural environment, through N leaching to groundwater, the runoff of surface water and emissions of hazardous gases, such us ammonium, nitrous oxide (N_2O) and nitric oxide (NO), as a result of soil microbial processes, nitrification and denitrification (Sanchez-Martin *et al*., 2008). The excessive use of fertilizers constitutes an environmental risk and, according to the European Commission, farming is responsible for over 50 % of total nitrogen discharges into surface waters. Nitrogen application is usually based on crop N requirements which are applied in accordance with the EU Nitrates Directive (Council Directive 91/676/EEC). In most EU countries, slurry application is limited to 170 kg N ha^{-1} $year^{-1}$. The original objective of the Nitrates Directive was to protect water quality across Europe by preventing nitrates from agricultural sources polluting ground and surface waters and by promoting the use of good farming practices. In line with this Council Directive, the regional government of Castilla-La Mancha (central Spain) published a set of guidelines for the N fertilization of crops, suggesting stricter limits in areas considered vulnerable to nitrate pollution (Real Decreto 261/1996, DOCM 2011: Orden 07/02/2011).

It is also important for crops to have a high level of Agronomic Efficiency. Nitrogen agronomic efficiency (AEN) is a parameter that expresses the ability of the plant to increase its yield in response to N application. Improving N use efficiency (NUE) in crops is also economically beneficial to producers and to the environment. A more efficient and ecologically responsible use of N could be achieved by adopting a series of improved strategies and management practices. Strategies commonly used to improve NUE include: genetic modification (new varieties that take up more organic or inorganic N from soil N and use absorbed N more efficiently); the selection of plant varieties with high harvest indexes; the genetic modification of amino acid transport; and cropping practice-based or crop management-based methods (crop

rotation with N-fixing crops and the optimization of the use of chemicals, biofertilizers, manures, and other N sources, tillage practices and cover cropping systems, etc.) (Perchlik and Tegeder, 2017).

Zinc is an essential micronutrient for plant nutrition and human health. However, Zn deficiency is a very widespread problem throughout the world and results in reduced crop yields and poor crop quality. About 50% of the soils used for cereal production in the world contain low levels of bioavailable Zn (Alloway 2008). Crop biofortification offers a sustainable and relatively cheap way to provide essential micronutrients to people in both developing and developed countries. Biofortification is a process that involves increasing the natural content of bioavailable nutrients in crop plants (Guo *et al*., 2016). The biofortification of cereals using Zn has been the focus of considerable research in recent years and has become increasingly relevant and important for both crops and humans (Guo *et al*., 2016). Applying Zn to barley increases both its fertility (number of grains per ear) and grain quality. Zinc concentrations and contents have been progressively increased through the application of Zn fertilization. In cases of extremely deficiency, Zn application may increase barley crop yields by as much as 48% compared with control crops only receiving NPK fertilizer (Singh 2008).

Zinc deficiencies in plants have traditionally been corrected by the application of inorganic Zn sources, such as ZnO, or salts, such as $ZnSO_4$ or $Zn(NO_3)_2$. Organic Zn sources, such as synthetic chelates and organic complexes, are now commonly used. Applications of Zn to calcareous soils in the form of chelates (such as Zn-EDTA –Zn- ethylenediaminetetraacetate-, Zn-DTPA –Zn- diethylenetriaminepentaacetate or Zn-HEDTA–Zn- hydroxyethyl-ethylenediaminetriacetate) can have important implications for crop nutrition and that these are highly effective sources of Zn for crops. Zinc chelates provide the micronutrients needed to produce high concentrations of water-soluble Zn and available Zn in soils, though the effectiveness of these chelates depends on their stability. However, Zn chelates differ in their physical state, chemical reactivity, cost, bioavailability and susceptibility to leaching. The chelating agents EDTA (ethylenediaminetetraacetate acid), HEDTA (hydroxyethyl-ethylenediaminetriacetate acid), and DTPA (diethylenetriaminepentaacetate acid) are some of the strongest synthetic chelating agents and when used in combination with Zn form much stronger chelates than naturally occurring organic ligands (Mortvedt and Gilkes, 1993). Zinc chelates enhance Zn availability due to less Zn^{2+} ion interaction with soil components (Pagani *et al*., 2013).

Various authors have studied the effects of applying Zn-N combinations using inorganic sources of Zn, such as ZnO, $ZnSO_4$ or $Zn(NO_3)_2$ (Cakmak *et al*., 2010; Erenoglu *et al*., 2011; Kutman *et al*., 2010). All these studies have focused on the impact of applying Zn-N combinations on N and Zn concentrations in plants in just one way: N fertilization combined with soil Zn applications has a significant interaction on grain Zn and N concentrations. Improving the N nutritional status of the plant may enhance the abundance of transporter proteins and nitrogenous chelators involved in the uptake, translocation, remobilization and grain allocation of Zn in cereals (Cakmak *et al*., 2010). For example, Kutman *et al* (2011) obtained increases of over 50% in Zn concentrations in whole grain wheat when the N supply was increased from low to very high. Zinc-N management appears to offer a promising agronomic strategy for the biofortification of cereals with Zn. On the other hand, to the best of our knowledge, there have been no specific studies of the application of Zn chelates in conjunction with N sources (organic *vs*. inorganic) to reduce the N rate with the aim of minimizing the environmental impact of fertilization yet maintaining adequate plant nutrient concentrations and a high crop yield.

The main objective of the present study was to determine the influence of the combined application of Zn, in the form of a synthesized Zn chelate fertilizer (Zn-DTPA- EDTA-HEDTA), and different N sources (pig slurry or urea) as a strategy to reduce the N application rate in a barley crop grown in a calcareous soil in order to use fertilizer more rationally and effectively and for improving both nitrogen use efficiency and Zn biofortification of barley.

The specific objectives were:

(1) to study the influence of Zn-DTPA-EDTA-HEDTA and N (pig slurry or urea) application at different rates on grain yield, Zn content and protein content in a barley crop

(2) to compare the agronomic efficiency of Zn and N applied via different combinations of Zn and N

(3) to establish the relative status of soil Zn fractions and the potential phytoavailability of soil-applied Zn based on different extraction methods

2. Material and Methods

2.1. Field locations

The field experiment was conducted at the "El Encín" Research Farm (40.52° N 3.30° W, 599 m) Madrid, Spain. This area is near "Alcarria-Guadalajara", which is in a part of Castilla-La Mancha that has been classified as sensitive to nitrate pollution contamination (DOCM, 2011). The average temperature from December to June was 1.1°C (average temperature range = 4.32–21.01°C). The rainfall in the crop growth period was 344 mm.

The soil at the site is classified as Calcic Haploxerept (Soil Survey Staff, 2010). The soil texture was silty-loam (31% Sand, 54% Silt and 15% Clay) with 8.20 pH, 188.1 µS cm^{-1} EC, 1.26% organic matter, 0.10% total nitrogen, 30.90 mg kg^{-1} extractable phosphorus, 51.70 mg total Zn kg^{-1} soil and 0.98 mg DPTA-TEA extractable Zn kg^{-1} soil.

2.2. Experimental design

The experiment was run with a randomised complete block design that included three replicate plots (7.5 m^2 $plot^{-1}$) for each treatment. Barley (Hordeum vulgare L. cv. Quench) was sown in December and harvested in early-June in the following year. Basal fertilization included 90 kg P_2O_5 ha^{-1} and 70 kg K_2O ha^{-1} (aqueous solutions of KH_2PO_4 and H_3PO_4).

A combination of three nitrogen (N) levels (30, 60 and 90 kg ha^{-1}) and four zinc (Zn) levels (0, 0.5, 1, and 1.5 kg ha^{-1}) of two different sources was applied during the early vegetative stage (February). Nitrogen was applied in the form of granular urea (46% N) or the liquid fraction of pig manure (total N, 2.0 g kg^{-1}; N-NH_4^+, 1.23 g kg^{-1}; total P, 97.5 mg kg^{-1}; total K, 1.32 g kg^{-1}, total Zn 22.14 mg kg^{-1}; total Cu 4.87 mg kg^{-1}; oxidizable organic matter, 4.60 g kg^{-1}; dissolved organic carbon, 0.5 g kg^{-1}; pH, 7,61 and DM, 16 g kg^{-1}). Zinc was applied as ZnCHE (Zn-DTPA-EDTA-HEDTA) [Zn-DPTA (Zn-diethylenetriaminepentaacetate), EDTA (Zn-ethylenediaminetetraacetate) and HEDTA (Zn-N-2-hydroxyethyl-ethylenediaminetriacetate)].

A control, without applications of either Zn or N fertilizer, was also performed (total plot number: 75) to calculate the NUE. This treatment was not used for statistical analysis as it did not conform to a real fertilization strategy used for this crop or in this region. Nitrogen fertilizer applications of about 100 kg N ha^{-1} are typically recommended for barley crops in this area (López-Bellido, 2009). The N application rates were therefore lower than typically recommended (with approximate reductions of 70%, 40% and 10%, respectively, compared to traditional application rates for the 30, 60 and 90 kg N ha^{-1} application rates).

2.3. Sample preparation and chemical analysis

A sample of six 0.5 m-long rows of barley plants were harvested from each plot. The different plant parts (root, stem, leaf and grain) were then separated. The root samples were carefully washed with deionized water and then all the samples were dried at 60 ºC for 72 h. Grain from the remaining plants was harvested using a plot combine (Weinterteiger Classic). Soil samples from the rhizosphere were collected and homogenised, air-dried, sieved (< 2mm) and stored for further analysis.

The Zn, Fe, Mn and Cu concentrations in the different parts of the plant (root, stem, leaf and grain) were determined by wet digestion in Sample Preparation Block Systems (SPB PROBE Perkin Elmer) using "Teflon tubes" kept at a maximum temperature of 150ºC for 2 hours, with 1.5 g of dried samples, 10 mL of HCl (37%), 10 mL of HNO_3 (65%) and 5 mL of HF (48%). The grain N concentration was determined using either the Kjeldahl or the Dumas method. Protein concentration was calculated as the N concentration multiplied by 5.83.

Total Zn, potentially available Zn (DTPA-TEA and Mehlich-3) and Zn distribution in the different soil fractions were all determined from the soil samples. Total Zn was determined by acid digestion after treating 1 g of soil with 14 mL HNO_3 (65%) and 6 mL HF (48%) followed by digestion in Teflon bombs in a microwave oven with a rotating tray. This involved a three-step process at a maximum pressure of 700 kPa.

The concentration of potentially available Zn in the soil was estimated using two different chemical extraction procedures: DTPA–TEA (Lindsay and Norvell, 1978) and Mehlich-3 (Tran and Simard, 1993). The distribution of Zn in the different fractions was obtained by selective sequential extraction (SSE). The SSE used was a six-step procedure which was performed according to Leleyter *et al*. (1999). The soil (g)/extractant solution (mL) ratio was 1:10. The Zn fractions were sequentially determined using a 2.5 g soil sample combined with the following extractants: NH_4Ac 1.0 M, pH=7.0 (Water soluble plus exchangeable, WSEX); NaOAc 1.0 M. pH = 4.5 (Carbonate bound, CAR); $NH_2OH{\cdot}HCl$ 0.1M in HNO_3, pH = 2.0 (Easily reducible oxide bound, RMO); $(NH_4)_2C_2O_4$ 0,2M + $H_2C_2O_4$ 0,2 M, pH = 3,0 (Amorphous minerals colloids bound, AMC); $(NH_4)_2C_2O_4$ 0.2 M + $H_2C_2O_4$ 0.2 M, pH = 3.0 in ascorbic acid 0.1 M (Crystalline Fe oxide bound, CFeO). The organic material and sulphide bound + residual fraction (OM + RES) was calculated as the difference between the total Zn extracted by wet acidic digestion in a microwave oven and the sum of the other fractions.

"Perkin-Elmer Pure" standard checks were used for the Quality Assurance System (certified by NIST–SRM). Standard solutions of Zn, Fe, Mn and Cu were prepared for each extraction in

background solutions of the extracting agents. In all cases, the Zn, Fe, Mn and Cu concentrations were determined by flame atomic absorption spectrophotometry (Perkin-Elmer AAnalyst 700).

2.4. Statistical analysis

Descriptive, simple and stepwise multiple regression analyses and other statistical studies were conducted using Statgraphics Plus software, version 5.1 (Manugistic Inc., Rockville, MD). Multifactor analyses of variance (ANOVA) of the parameters studied were carried out to determine the main effects of the Zn application rate, N application rate, N source and experimental repetition, and the interactions between them. When two-way interaction between the Zn rate and N rate factors was significant, we performed a new multifactor analysis of variance to determine the main effects of the combined factor. Means were compared using Fisher's protected least significant difference (LSD) tests. Differences were considered significant at $P < 0.05$.

3. Results

3.1. Plant response to fertilizer treatments

The analysis of variance (ANOVA) for the plant variables studied in the barley crop to determine the main effects of the ZnCHE (Zn-DTPA-EDTA-HEDTA) application rate (ZnCHE-0, ZnCHE-0.5, ZnCHE-1, ZnCHE-1.5), N application rate (N30, N60, N90) and N source (pig slurry –PS- or urea) and the interaction between these main effects are shown in Table 1. The Zn and N application rates caused significant differences in grain yield and Zn concentrations in the different parts of plant. However, only grain yield was significantly influenced by the N source. In leaf and grain Zn concentrations, there were interactions between the Zn application rate and N source. In the grain Zn concentration there was also interaction between the N application rate and N source.

(Table 1)

Figure 1 shows the grain yield for each treatment and also the mean values when all the data were studied in combination. The highest grain yield was observed with PS + N90 treatments

when ZnCHE was applied at any of the three Zn application rates (3873, 3857 and 3928 kg ha^{-1}, with ZnCHE-0.5, ZnCHE-1.0 and ZnCHE-1.5, respectively). The lowest grain yield was obtained without a Zn treatment (ZnCHE-0) and with N30, applied either as PS or in the form of urea treatments (3015 and 3026 kg ha^{-1}, respectively). According to the statistics, grain yield increased when all of the ZnCHE application treatments were compared to the ZnCHE-0 treatments (by between 9.5 and 11.0% compared to the ZnCHE-0 treatments). An increase in the N application rate also significantly increased the mean grain yield (by between 6.9 and 13.0%) and the application of PS produced a higher mean grain yield than urea (which increased it by 6.0%). Orthogonal contrasts were used to compare the effects of the combined application of Zn and N on mean grain yield. Increases were observed in the mean grain yield produced by combined application of Zn and N compared with the combined application of the nil-Zn treatment (ZnCHE-0) and N. The estimated difference between the treatment means was 1505 kg ha^{-1} for ZnCHE-0 (N30+N60+N90) *vs.* ZnCHE-0.5 + ZnCHE-1.0 + ZnCHE-1.5 (N30+N60+N90) ($P < 0.05$).

(Figure1)

The mean Zn concentrations in root, stem, leaf and grain obtained with the different Zn and N application rates and N sources and their respective interactions are shown in Table 2. The different Zn rates employed had an effect on the mean Zn concentrations in the different parts of the plant (root, stem, leaf and grain), with average increases of up to 75, 106, 59 and 30%, respectively, when the ZnCHE-1.5 treatments were compared to the ZnCHE-0 treatments.

(Table 2)

Increasing the N application rate from N30 to N60 significantly increased the mean Zn concentrations in the root and stem (by 10.2 and 26.0%, respectively). In contrast, the mean Zn concentrations in the root and stem were not significantly affected by an increase in the N application rate from N60 to N90. However, increases in the N application rate from N30 to N60 and from N60 to N90 significantly increased the mean Zn concentrations in the leaf (by 8.2 and 14.7%) and grain (by 7.8% and 8.0%), respectively. In contrast, the different N sources employed (PS or urea) had no effect on the mean Zn concentrations in the different parts of the plant.

The combined factor of N sources × Zn application rates significantly affected ($P < 0.0001$) the leaf and grain Zn concentrations. The treatments in which ZnCHE-1.5 was applied (both with urea and with PS), and also those involving PS + ZnCHE-1.0, produced the highest leaf and grain Zn concentrations. The combined factor of N sources × N application rates employed only

had a significant ($P < 0.05$) effect on grain Zn concentration. The highest grain Zn concentrations were observed with the urea + N90 treatment. Even so, there were no significant differences between the grain Zn concentration obtained with the urea + N90 treatment and the PS + N90 and PS + N60 treatments.

The grain N and Zn uptake and grain protein concentrations obtained with each of the treatments are shown in Fig. 2. As expected, the mean comparisons for the N application showed that N uptake significantly ($P < 0.0001$) increased with each increment in the N application rate; it increased by 26.9% with an increase in the N application rate from N30 to N60 and by 16.4% with an increase from N60 to N90. The Zn rate administered also significantly influenced ($P < 0.0001$) the N uptake. An increase in the mean N uptake was observed when the Zn application rate was increased from ZnCHE-0 to ZnCHE-0.5 (with an increase of 32.6% in the mean N uptake) and from ZnCHE-0.5 to ZnCHE-1.0 (with an increase of 11.8% in the mean N uptake). There were, however, no significant differences between the N uptake obtained with the ZnCHE-1.0 and ZnCHE-1.5 treatments.

(Figure 2)

As expected, the Zn uptake significantly increased ($P < 0.0001$) with increases in the Zn application rate. Increases of 23.1 and 7.2% were observed in mean Zn uptake respectively, when the PS + ZnCHE-0.5 and urea + ZnCHE-0.5 treatments were compared to the ZnCHE-0 treatments. Moreover, increases of 59.4 and 25.9% were observed in the mean Zn uptake by changing from ZnCHE-0 to ZnCHE-1.5, applying PS and urea, respectively. Furthermore, increases in the N application rate significantly ($P < 0.0001$) increased the Zn uptake (with increases of 12.3% and 19.0% in the mean Zn uptake when the N application rate was increased from N30 to N60, applying PS and urea, respectively, or with increases of 22.2% and 46.6% in the mean Zn uptake when the N application rate was increased from N30 to N90, applying PS and urea, respectively).

An increase in the N application rate significantly increased mean grain protein concentrations. On the other hand, applying ZnCHE produced significantly ($P < 0.0001$) higher mean grain protein concentrations than the ZnCHE-0 treatments. Increases in mean grain protein concentrations of from 21.4% to 34.8% were observed when applying ZnCHE as opposed to ZnCHE-0 treatments. The protein concentration was not significantly affected by an increase in the Zn application rate from ZnCHE-1 to ZnCHE-1.5. Something similar occurred with total N uptake by grain, although the grain protein concentration was significantly affected by the

different N sources employed, with higher mean grain protein concentrations being associated with treatments using urea than those applying PS.

The Fe, Mn and Cu concentrations in several aerial parts of the plant (stem, leaf and grain) are shown in Figure 3. In the case of stem Fe concentration, significant ($P < 0.0001$) differences were observed between N sources. Greater increases in Fe levels were observed in the stem with PS applications than with urea treatments (22.98 and 16.67 mg Fe kg^{-1}, respectively). Significant differences (the *P values* ranged from 0.0000 to 0.05) were observed between N application rates or N sources in leaf and grain Fe concentrations. As the statistical interaction between the N source and N application rate factors were significant ($P < 0.05$) for leaf and grain concentrations, we used a factor that combined both of them: N source × N application rates. Significant ($P < 0.0001$) differences were observed between the N source × N application rates factor in terms of Fe concentration in these parts of the plant. The highest concentrations were observed for the treatment with the highest N application rate (N90) and where PS was applied. Even so, there were no significant differences between the leaf or grain Zn concentrations obtained with PS + N30 and any of the urea treatments. Furthermore, significant differences ($P < 0.05$) were observed between N application rates or N sources in stem Mn concentrations. Significant differences were also observed between the N source × N application rates interaction in the stem Mn concentration. The highest mean concentrations were observed for the treatment with the highest N application rate (N90) and where PS was applied.

(Figure 3)

3.2. Zinc, Fe, Mn and Cu status of the soil

The effect of the fertilizer treatment on the total Zn, DTPA-TEA- and Mehlich-3-extractable Zn and Zn fractions in the soil at harvest are shown in Table 3. In total and available (Zn-DTPA-TEA and Zn-Mehlich-3) Zn concentrations significant differences were observed between Zn application rates. Significant differences were also observed between Zn application rates in all of the fractions except the CFeO, OM and RES fractions. Zinc and N application rates and N source factors did not show any statistically significant interactions in any of the cases studied. As expected, when the Zn concentrations in the soil were statistically different, the highest Zn concentrations were observed with ZnCHE applied at 1.5 mg Zn kg^{-1} and the lowest Zn concentrations were obtained with ZnCHE-0.

(Table 3)

The total available Fe, Mn and Cu concentrations in the soil (DTPA-TEA and Mehlich-3) and distributions within the different soil fractions did not show any significant differences between Zn application rates (data not shown). Potentially available (DTPA-TEA and Mehlich-3) Fe concentrations in the soil showed significant ($P < 0.05$) differences between N application rates or N sources. Furthermore, Fe-WSEX+CAR showed significant ($P < 0.001$) differences between N application rates or N sources. The statistical interaction between N source and the N application rates factors were significant ($P < 0.05$) in all these cases. Significant (P between 0.008 and 0.046) differences were observed between N source × the N application rates interaction for Fe concentration in the soil. The highest mean concentrations were observed for the treatment with the highest N application rate (N90) and where PS was applied (Fe-DTPA-TEA, 5.08 mg Fe kg^{-1}; Fe-Mehlich-3, 42.90 mg Fe kg^{-1}; Fe-WSEX+CAR, 2.12 mg Fe kg^{-1}). The lowest mean concentrations were observed for the treatments including urea (Fe-DTPA-TEA $_{urea + N60}$ = 4.26 mg Fe kg^{-1}; Fe-Mehlich-3 $_{urea + N30}$ = 30.80 mg Fe kg^{-1}; Fe-WSEX+CAR $_{urea + N60}$ = 1.27 mg Fe kg^{-1}). Even so, there were no significant differences between the potentially available Fe concentration obtained with PS + N30 and the urea treatments.

On the other hand, Mn-Mehlich-3 showed significant ($P < 0.05$) differences between N application rates or N sources. The statistical interaction between N source and the N application rate factors was significant ($P < 0.05$). The Mn-Mehlich-3 concentrations ranged from 61.75 mg Mn kg^{-1} to 76.08 mg Mn kg^{-1}, urea + N60 and PS + N90, respectively. Conversely, the potentially available (DTPA-TEA and Mehlich-3) Cu concentration in the soil and its distribution within the different soil fractions did not show any significant differences between N sources.

4. Discussion

4.1. Influence of combined application of Zn and N on yield, biofortification and plant quality

There was an improvement in grain yield when ZnCHE fertilizer was added. This enhancement (9-11%) was similar to the mean yield increase observed when the N rate was increased from N30 to N60 or N90 (7-13%) (see Fig. 1). This improvement, which was evident following the application of Zn fertilization, could be attributed to an adequate Zn supply that might have

affected metabolic and enzymatic activity. There were no significant differences between the grain yields obtained with the different treatments in which Zn was applied. The mean yields obtained with the combined applications of Zn and N were greater than those obtained when applying only N. Harapiak *et al* (2000) reported that, in general, the barley yield response to micronutrients was 3% of the total response on the Canadian Prairies, while N was responsible for 73 % of the increase in yield. Nitrogen is the key element for achieving high cereal yields. In our experiment, grain yield increased with the N application rate (e.g. the application of N90 + ZnCHE-0 increases 23% and 12.5% in mean grain yield respect to the treatment N30 + ZnCHE-0, for PS and urea, respectively). Plots fertilized using the lowest N application rate produced the lowest grain yields. Limited N availability affects the supply of carbohydrates to developing seeds and thereby reduces grain weight.

On the other hand, the application of PS rather than urea produced higher mean grain yields (see Fig.1). Increases in the N + Zn application rates had a great impact on grain yield, with mean values increasing by up to 30.3% with PS and by 23.1% with urea. This could have been due to an increase in the available nutrients and dissolved organic carbon (DOC) to the crop through the increase in their supply with the application of pig slurry. In our study, significant correlations and positive coefficients were observed between grain yield and the Zn, Fe and Cu concentrations in grain ($P < 0.05$, n = 72). Zeidan *et al* (2010) reported increases in grain yield with respect to the control for the application of Zn and Fe of 42% and 40%, respectively, in a wheat crop.

In our experiment, the combined application of N and Zn also had a significant, positive effect on grain Zn concentrations (see Table 2). The use of agronomic strategies to increase concentrations of mineral elements in edible plants is commonly known as Agronomic Biofortification. In countries whose populations exhibit a high incidence of micronutrient deficiencies, cereal-based foods generally represent a considerable proportion of the daily diet (Cakmak *et al*., 2010). According to Velu *et al*. (2014), the Zn concentration in wheat in many countries ranges from 20 to 35 mg kg^{-1}. In our study, we observed a total Zn concentration in barley of between 37 and 72.4 mg Zn kg^{-1} and in grain we found Zn concentrations of between 17.4 and 27.5 mg Zn kg^{-1} (PS × N30 + ZnCHE-0 and Urea × N90 + ZnCHE-1.5, respectively). Erenoglu *et al*. (2011) and Kutman *et al*. (2010) observed that the N nutritional status of wheat plants affects root uptake and shoot transport, retranslocation from vegetative tissues to seeds and the seed allocation of Zn. The redistribution of Zn from leaves to sinks and from roots to shoots is a mechanism that makes it possible to provide emerging organs and maturing seeds

with appropriate concentrations of Zn in all cereals. Various authors have studied the mobility of metals within plants by calculating the TF (translocation factor); this is defined as the relationship between the micronutrient concentrations in two different parts of a given plant (Kabata and Pendias, 2001; Podlesakova et al., 2001). A high TF value indicates greater mobility (Intawongse and Dean, 2006; Almendros et al., 2015).

In our study, significant differences in TF were noted for the different Zn treatments ($P < 0.0001$ in TF grain/leaf, TF grain/stem and TF grain/root; $P < 0.001$ in TF leaf/stem and $P < 0.05$ in TF stem/root) and N rates applied ($P < 0.05$ in TF stem/root, TF grain/stem and TF leaf/stem). The highest TF stem/root values were observed with the ZnCHE-1.5 and N60 or N90 treatments. Kutman *et al*. (2010) reported that the positive interaction between N and Zn in cereals is due to improvements in root uptake and the translocation of Zn due to the presence of N. Several nitrogenous compounds, such as amino acids and nicotianamine, could be involved in the translocation of Zn from the root into the shoot. However, in our study it was observed that the TF reached similar values with the N60 and N90 treatments when the highest Zn rate was added (ZnCHE-1.5).

In contrast, for TF grain/leaf, TF grain/stem, TF grain/root ($P < 0.0001$) and TF leaf/stem ($P < 0.001$) the highest values recorded in our study were observed with the ZnCHE-0 treatments. This seems to suggest that when the Zn concentration in plant is low, it assures the accumulation of Zn in grain or leaves.

In our experiment, N application rates did not cause any significant differences in TF grain/leaf. These results differed from those reported by Erenoglu *et al*. (2011) who, in a radiolabelled experiment conducted with ^{65}Zn and carried out in wheat, found that plants with high levels of N translocated more ^{65}Zn from flag leaves into seeds than those containing low levels of N. This difference could be explained by the fact that the N doses applied in our experiment were low (with approximate reductions of 70%, 40% and 10% compared to the traditional application rates used in our area).

The Zn uptake in plants significantly increased at both of the Zn and N application rates (see Fig 2). As Zn uptake is calculated as the product of grain Zn concentration and yield, the highest values for this crop were obtained with applications of PS. Combined applications of Zn and N also significantly increase the grain protein concentration and N uptake. Various authors have reported increases in protein content associated with combined applications of N and Zn, in different crops: rice (Guo *et al*., 2016), maize (Sajad *et al*., 2014), wheat (Cakmak *et al*., 2010) and fodder sorghum (Verma *et al*., 2005). Speciation and localization studies conducted on

456 cereal grain indicate that Zn interacts with proteins in the grain and therefore grain proteins
457 constitute a physiological sink for Zn (Cakmak and Kutman, 2018). Zinc plays an indispensable
458 role in protein synthesis and Zn deficiency impedes this process and affects the health and
459 productivity of plants, resulting in low yields and poor plant quality. According to FEDNA
460 (2016), grain protein concentrations of between 9.6 ("lower protein level") and 11.3% ("upper
461 protein level") in barley grain are appropriate for feed use. In Spain, average analyses for the
462 last six years show a tendency towards a reduction in the protein content of barley grain and an
463 increase in the concentration of starch; this is probably due to the fact that N application has
464 been limited by the Nitrates Directive. In our study, all the treatments with N90 reached these
465 values when ZnCHE was applied (see Fig. 2). Moreover, treatments with N60 and ZnCHE-1
466 or ZnCHE-1.5 also achieved this range. Conversely, the only treatment involving the lowest N
467 rate applied to reach this range was the urea × N30 + ZnCHE-1.5 treatment. It is noteworthy
468 that in our study PS application affected the grain protein concentration, which decreased by
469 10% when PS was applied instead of urea. This may reflect a common characteristic of pig
470 slurry: once it has been added to soil, the ammoniacal N in slurry quickly changes into nitric N
471 and this may leach if plants do not assimilate it (Sanchez and Gonzalez 2005). Moreover, N
472 from manure is easily lost via gaseous emissions (NH_3, N_2O and NO).
473
474 *4.2. Influence of treatment added on the efficiency of Zn or N use*.
475
476 To evaluate the relative effectiveness of the different treatments, we established the agronomic
477 efficiency of Zn or N use (AEZn and AEN, respectively). These parameters were used to study
478 the capacity of yield increase per kg of Zn or N applied. Significant differences between N or
479 Zn application rates were observed in AEN (P between 0.009 and 0.0000). The factors studied
480 did not show any statistically significant interactions in any of the cases. As shown in Figure 4,
481 it was possible to observe a (non-significant) tendency for AEZn to decrease with increases in
482 the application rates of Zn and N and also with urea application. However, the mean AEN
483 obtained with the application of Zn fertilizer was significantly ($P < 0.001$) greater than that
484 obtained by only applying N. According to Cakmak (2010), N and Zn act synergistically when
485 they are both present in sufficient amounts. Arora and Singh (2004) reported the beneficial
486 effect of Zn application on N availability, possibly as a result of the activation of a physiological
487 process in which Zn acts as a co-enzyme and/or catalyst. Since the agronomic efficiency of N

use (AEN) describes the capacity to increase the yield per kg of N applied, the highest AEN values for this crop were obtained with applications of the lowest N doses (N30 treatments). These results are in line with Tilman *et al*., (2002) who reported the highest efficiency of N fertilizer use being associated with the first increments in added N, with AEN decreasing with higher levels of N application.

(Figure 4)

According to Prasad and Sinha (1981), the percentage of Zn used, or Zn utilization, by the crop is a decisive parameter for the relative effectiveness of any fertilizer treatment. In our study, significant differences were observed between N sources ($P < 0.0001$) or Zn application rates ($P < 0.05$). The factors studied did not show any statistically significant interactions in Zn or N utilization. As expected, in this crop the highest Zn utilization values were obtained with applications of the highest Zn doses (ZnCHE-1.5 treatments). There were differences between the results obtained from treatments with ZnCHE-1.0 and ZnCHE-1.5, but these were not significant. In addition, the application of Zn fertilizer produced significant ($P < 0.0001$) increases in N utilization with respect to the control treatment. The highest N utilization values were obtained with applications of the ZnCHE-1.0 and ZnCHE-1.5 treatments. According to several studies (Hu *et al*., 2003; Montoya *et al*., 2018), ethylenediamine-based chelating agents, such as DTPA and EDTA, may act as nitrification inhibitors. Nitrification inhibition caused by heavy metals, such as Zn, has also been addressed in the literature (Kapoor *et al*., 2015). The application of nitrification inhibitors is a strategy employed to increase the efficiency of N use in crops.

The highest level of N utilization was obtained with the lowest N doses (N30 treatments); this result was in line with the AEN. The differences in Zn and N utilization between the different N sources applied (PS and urea) were consistent with the results obtained for Zn uptake in plant and grain protein concentration, which was, in itself, directly related to the N concentration in the plant.

4.3. Soil status and nutrient availability to the plant

The amount of Zn in soils and plants depends on the source applied, its behaviour (fixation, leaching, transformation, availability) and several other plant parameters (uptake, transport and accumulation). Various authors have assessed the mobility of metals by calculating soil-to-plant

transfer factors (TF_{S-P}) (Kabata-Pendias, 2001; Prasad and Sinh, 1981) this concept is defined as the relationship between the Zn concentration in the plant root and the total concentration of this element in the soil. A high TF_{S-P} value indicates greater soil-to-plant mobility and availability, while a low value indicates a greater probability of there being a deficiency (Alloway, 2008). In our study, significant differences between N application rates ($P < 0.0001$) or Zn application rates ($P < 0.0001$) were obtained for TF_{S-P}. The mean TF_{S-P} concentration increased with N application rates in the following order: N30 (0.31) < N60 (0.34) ≈ N90 (0.35). Chaudhry *et al*. (1977) and Singh and Singh (1981) observed higher Zn contents in plant tissues as a result of applying more N in response to an increase in Zn solubility. In contrast, the mean TF_{S-P} concentration increased in the following order: ZnCHE-0 (0.24) < ZnCHE-0.5 (0.31) < ZnCHE-1 (0.38) < ZnCHE-1.5 (0.41). This Zn source: ZnCHE (Zn-DTPA-EDTA-HEDTA), was associated with large amounts of bioavailable Zn in the soil and also with high Zn concentrations resulting from the sum of the most labile fractions (water soluble plus exchangeable fractions) in the soil with similar properties (Almendros *et al*., 2015). This behaviour could be related to differences in the stability constant (K) of the chelates studied under these soil and plant conditions. The Zn sources that contain the most stable chelates (log $K_{Zn\text{-}EDTA} = 17.5$ and log $K_{Zn\text{-}DTPA} = 19.5$ with an ionic strength of 0.01 mol L^{-1}) (Lindsay, 1979; Smith *et al*., 2004) maintain greater amounts of Zn in their soil solution. In contrast, in Zn sources that contain fewer stable chelates, for example Zn-HEDTA (log $K_{Zn\text{-}HEDTA} = 15.3$ with an ionic strength of 0.01 mol L^{-1}), the metal is retained by the soil components.

5. Conclusion

Nitrogen-Zn interaction influences both plant yield and its nutritional composition. The application of Zn is necessary in two ways: to increase the Zn concentration in the plant, and because it also affects the amount of N in the plant. A judicious use of Zn and N in barley crops could result in better crop production in both economic and sustainable terms. An application rate of 0.5 mg Zn kg^{-1} (ZnCHE-0.5) seems sufficient to achieve a great grain yield. However, it is recommendable to increase the dose of Zn applied in order to reach high nutritional values as this improves the Zn concentration in the plant or grain protein concentration. Higher Zn doses are also advisable to achieve high levels of Zn and N uptake in plants and to increase root-stem Zn translocation. These effects are probably due not only to Zn application, but also

552 to the possible influence of chelating agents, such as DTPA, HEDTA and EDTA, as nitrification
553 inhibitors. In contrast, the 90 kg N ha^{-1} application rate would seem recommendable under these
554 conditions since it was associated with high values in terms of crop yield, grain protein
555 concentration and plant Zn and N uptake. These treatments would imply a reduction of 25% in
556 the "rational N application rate" for this crop under these dry-land conditions. Pig slurry
557 application produced a higher mean grain yield, Zn uptake and Zn utilization but a lower grain
558 protein concentration than urea applications. This could be due, on the one hand, to an increase
559 in the available nutrients to the crop through the increase in their supply with pig slurry and/or,
560 on the other hand, to the different behaviour of the forms of N applied to the soil (N losses and
561 availability).
562
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565
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Table 1: Analysis of variance (ANOVA) of the effects of Zn application rate, N application rate, N source and their interactions on grain yield; root, stem, leaf and grain Zn concentrations; N and Zn uptake and grain protein concentration

	Zn application rate (a)	N application rate (b)	N source (c)	a × b	a × c	b × c	a × b × c
df	3	2	1	6	3	2	6
Grain yield	< 0.05	< 0.05	< 0.05	NS	NS	NS	NS
Root Zn conc	< 0.0001	< 0.001	NS	NS	NS	NS	NS
Stem Zn conc	< 0.0001	< 0.0001	NS	NS	NS	NS	NS
Leaf Zn conc	< 0.0001	< 0.0001	NS	NS	< 0.05	NS	NS
Grain Zn conc	< 0.0001	< 0.0001	NS	NS	< 0.05	< 0.05	NS
N uptake	< 0.0001	< 0.0001	NS	NS	NS	NS	NS
Zn uptake	< 0.0001	< 0.0001	< 0.05	NS	< 0.05	< 0.05	NS
Grain protein conc	< 0.0001	< 0.0001	< 0.001	NS	NS	NS	NS

NS, not significant

Table 2: Zinc concentrations in root, stem, leaf and grain obtained with the different Zn and N application rates and N sources. Italic values show mean Zn concentrations and the ANOVA of the effects of Zn application rate, N application rate, N source or their interactions when these were significant.

		ROOT [Zn] mg/kg			STEM [Zn] mg/kg			LEAF [Zn] mg/kg			GRAIN [Zn] mg/kg		
	Zn	N30	N60	N90	N30	N60	N90	N30	N60	N90	N30	N60	N90
PS	0	10.35	11.95	13.66	3.29	4.29	4.56	6.01	6.30	7.00	17.36	18.41	19.08
	0.5	14.66	16.86	17.08	4.56	5.98	6.45	7.27	7.61	8.91	19.74	20.25	20.91
	1	20.85	21.46	22.60	5.99	6.52	7.07	9.23	9.92	12.16	23.35	24.07	25.02
	1.5	21.10	22.92	23.89	7.19	8.17	9.22	8.98	9.84	12.28	24.38	26.23	27.01
UREA	0	12.47	13.69	14.00	3.36	3.98	4.15	5.98	6.86	7.04	17.86	19.95	21.98
	0.5	13.74	18.50	18.00	4.22	5.52	5.58	7.72	7.80	10.53	18.47	20.25	22.69
	1	19.41	19.75	19.98	5.36	7.19	7.29	7.96	9.17	10.23	18.71	21.13	24.39
	1.5	20.55	21.54	23.05	6.30	9.07	8.69	10.07	10.90	10.29	21.03	23.04	27.47

							PS × Zn0	*18.28 a*
						N30	*7.90 a*	*PS ×Zn0.5*	*20.30 ab*
Zn-0	*12.69 a*	*Zn-0*	*3.94 a*			*N60*	*8.55 b*	*PS ×Zn1*	*24.14 c*
Zn-0.5	*16.47 b*	*Zn-0.5*	*5.39 b*			*N90*	*9.81 c*	*PS ×Zn1.5*	*25.87 c*
Zn-1	*20.67 c*	*Zn-1*	*6.57 c*					*U × Zn0*	*19.93 ab*
Zn-1.5	*22.17 d*	*Zn-1.5*	*8.11 d*			*PS × Zn0*	*6.44 a*	*U ×Zn0.5*	*20.01 ab*
						PS	*7.93 b*	*U ×Zn1*	*21.41 b*
N30	*16.64 a*	*N30*	*5.03 a*			*PS ×Zn1*	*10.44 c*	*U ×Zn1.5*	*23.84 c*
N60	*18.33 b*	*N60*	*6.34 b*			*PS*	*10.37 c*		
N90	*19.03 b*	*N90*	*6.63 b*			*U × Zn0*	*6.63 a*	*PS × N30*	*21.21 ab*
						U	*8.69 b*	*PS ×N60*	*22.24 bc*
PS	*18.11 a*	*PS*	*6.11 a*			*U ×Zn1*	*9.12 b*	*PS ×N90*	*23.00 bc*
UREA	*17.89 a*	*UREA*	*5.89 a*			*U*	*10.42 c*	*U × N30*	*19.02 a*
								U ×N60	*21.09 ab*
								U ×N90	*23.79 c*

Values compared using LSD multiple-range test at the 0.05 level of probability. Homogeneous groups are denoted with the same letter

679 **Table 3**: Analysis of variance (ANOVA) and effect of fertilizer treatment on total Zn, DTPA-
680 TEA, and Mehlich-3 extractable Zn and Zn fractions in soil (mg kg^{-1}) at the time of barley
681 harvest
682

	Total Zn	Zn-DTPA-TEA	Zn-Mehlich-3	Zn-WSEX	Zn-CAR	Zn-RMO	Zn-AMC	Zn-CFeO	Zn-OM + Zn-RES
Source of variation									
Zn application rate (a)	**	**	**	***	***	***	**	ns	ns
N application rate (b)	ns	ns	ns	ns	ns	ns	ns	ns	ns
N source (c)	ns	ns	ns	ns	ns	ns	ns	ns	ns
ZnCHE									
0	52.69 a	1.00 a	2.28 a	0.28 a	1.00 a	0.61 a	3.72 a	1.25 ab	46.49 a
0.5	53.50 b	1.10 ab	2.36 ab	0.29 ab	1.00 a	0.68 a	4.68 b	1.17 a	49.89 a
1	53.90 bc	1.18 bc	2.41 b	0.31 b	1.30 b	0.84 b	4.94 bc	1.18 a	49.70 a
1.5	54.30 c	1.29 c	2.47 b	0.34 c	1.41 b	0.92 b	5.18 c	1.16 a	51.33 a

683
684
685 *** . ** and * significant at 0.01 %, 0.1 % and 5 % levels. Values compared using LSD
686 multiple-range test at the 0.05 level of probability. Homogeneous groups are denoted with the
687 same letter.
688
689

690 **Figure 1.** Effect of the different fertilizer treatment on grain yield. The vertical line at each of
691 the data represents the standard deviation from the mean. Data table shows the mean values
692 when all the data were studied in combination. Mean values followed by different letters are
693 significantly different at $P \leq 0.05$ by the LSD test.

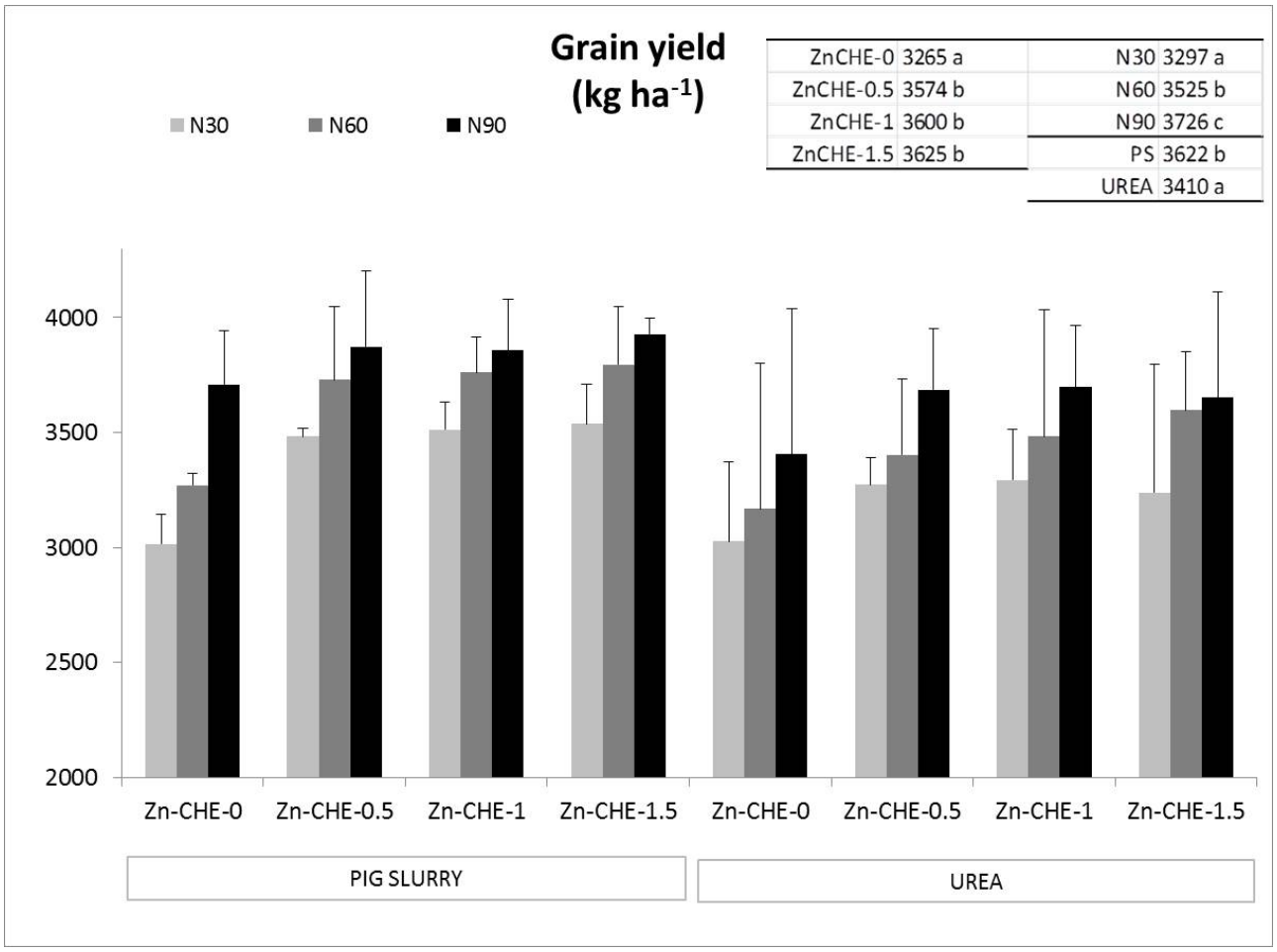

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Figure 2. N and Zn uptake and grain protein concentrations obtained with each treatment. The vertical line at each of the data represents the standard deviation from the mean. Data table shows the mean values when all the data were studied in combination (N uptake, roman letters; Zn uptake, italic letters; grain protein concentration, bolt letters). Mean values followed by different letters are significantly different at $P \leq 0.05$ by the LSD test.

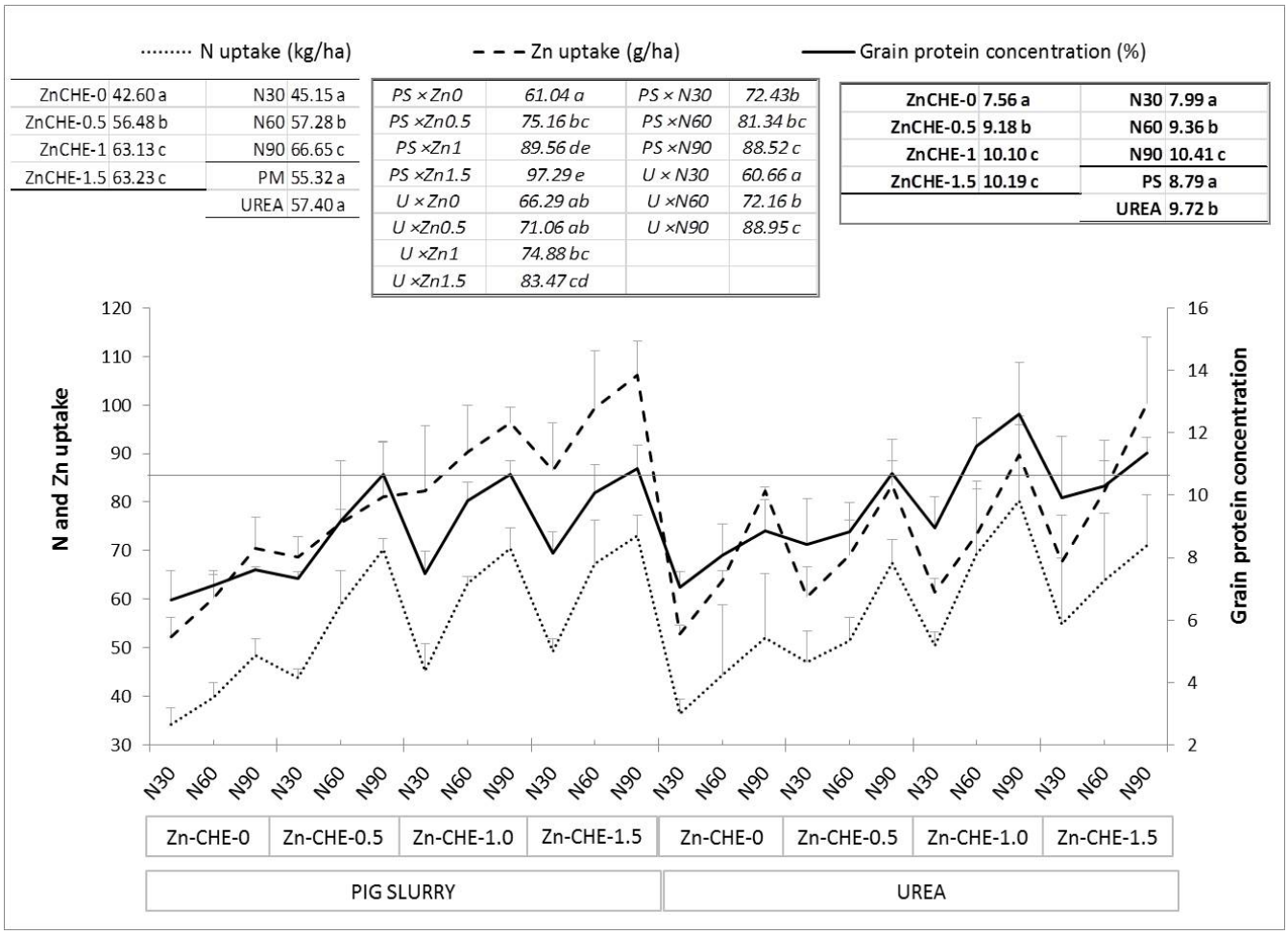

Figure 3. Fe, Mn and Cu concentrations in stem, leaf and grain, obtained with each treatment. The vertical line at each of the data represents the standard deviation from the mean. Data table shows the mean values when all the data were studied in combination. Mean values followed by different letters are significantly different at $P \leq 0.05$ by the LSD test.

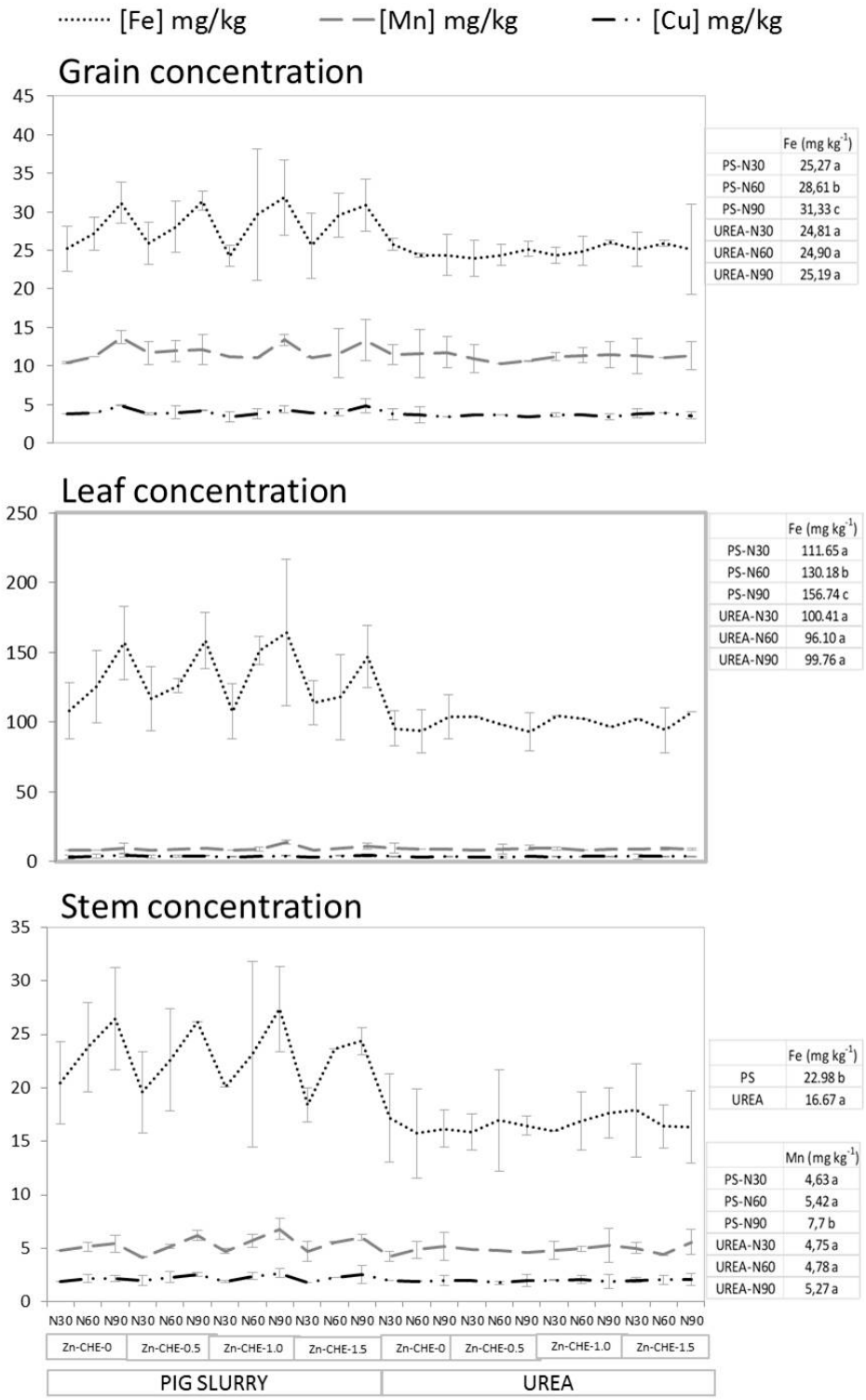

Figure 4. Agronomic efficiency of Zn or N use (AEZn and AEN, respectively), Zn and N utilization obtained with each treatment. The vertical line at each of the data represents the standard deviation from the mean. Data table shows the mean values when all the data were studied in combination. Mean values followed by different letters are significantly different at $P \leq 0.05$ by the LSD test.