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RESEARCH ARTICLE

Wheat plant response to zinc enrichment: results from a big plot assay

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ABSTRACT

One third of the global population suffers Zn deficiency, which directly affects their health and the health-bill of all world countries. In this study, the application of Zn sulphate at the latest developing stages (anthesis and milk-dough stages) of bread wheat in Mediterranean conditions were tested for grain and leaves enrichment capacity and antioxidant activities prompting. Variety effects were found to be significant to success in enriching leaves and grains with Zn. While Almansor and Roxo varieties increased more than 50% and close to 40% respectively their amount in grain Zn, no significant differences were found for Paiva variety with or without Zn treatment. Regarding the leaves, Zn amount increases of 110, 230 and 300 ppm of Zn were figured out in Almansor, Roxo and Paiva varieties respectively. Antioxidant compounds in leaves showed to be higher when zinc treatment was applied in Almansor variety. Quality traits of the grain wheat were barely affected by the increase of the Zn amount; Roxo variety grains increased the protein and the dry gluten concentration, while Almansor grains were heavier and Almansor flour showed higher tenacity. It can be concluded that Zn enrichment in wheat plants caused variation in both grain and leaves mineral profile, and antioxidants in leaves.

Keywords: Antioxidants; Rainfed conditions; RXF; Winter cereals; Zinc

INTRODUCTION

In 2003, WHO and FAO in their together report indicated that almost half of the world population, mainly in developing countries, were affected by micronutrients deficiency (WHO, 2003a). Nowadays, in 2022, the situation has not improved a lot; nearly one-third of people worldwide suffer from at least one form of micronutrient deficiency (HLPE, 2017; Global Nutrition Report, 2020).

Such micronutrient deficiency, regarding especially to Fe, Zn or vitamin A, is a warning global health risk, due to the key role that micronutrients play in human body system (Dary and Hurrell, 2006). But not only developing countries are suffering this "*hidden hunger*" problem; every day more cases of low dietary intake of micronutrients are reported in Europe, especially in both elderly people and children. In these two age groups deficiencies on Zn, Fe, Se or I, can affect several critical functions including cognition, immune response, thyroid function, or stunted growth and development (Bailey et al., 2015; Steinbrenner et al., 2015; Vreugdenhil et al., 2021). Recently, after the appearance of the coronavirus global pandemic, it has been proven that a correct Zn level in blood results in a decrease in the severity and mortality of COVID-19 (Ali et al., 2021). This could be due, among others, to the immunoregulatory and antiviral properties that Zn exhibits; in fact, the WHO had previously related Zn deficiency to about 16% of the all respiratory diseases worldwide (WHO, 2003b).

Knowing that a low amount of mineral elements in the soil implies a low plant intake in such minerals (Broadley et al., 2010), this indicates a direct connection between the plant mineral profile and health population) Ahmed et al., 2022). Therefore, the current pressure to feed and nourish an ever-growing human population using the same (or even less) arable land and fresh water, makes agronomical techniques leading to get more/better products a key factor to achieve such an important goal (Basarir et al., 2022). This fact acquires especial importance in people

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with plant-basel diets where cereals-derived foods are usually the most consumed (Harding et al., 2018; Van Der Straeten et al., 2020).

In this regard, it has been shown that enriching crops with minerals is an important tool to get better quality raw material in a simple, environmentally friendly and cost-effective way (Wakeel and Labuschagne, 2021) helping to increase micronutrients intake in world population and having significant positive effect on human health (Bouis and Saltzman, 2017; Praharaj et al., 2021).

Specifically, numerous works have been developed to determine the best agricultural techniques to increase Zn in wheat crop, due to the previously mentioned importance of the Zn in human health and the great importance of the wheat crop (counted among the "*big three*" cereal crops harvested worldwide). Thus, it has been determined that type of application (foliar vs. soil), soil type, product used in the program, or the combination with another fertilizers, as well as the timing, can contribute to the success of the Zn enriching program in wheat (Cakmak, 2018; White and Broadley, 2008; Cakmak et al., 2010; Jalal et al., 2020). Nice approaches to a proper enriching program in wheat in Mediterranean areas, with such a particular climate highlighting the water stress in the latest crop developing stages, have been published by Luís et al. 82021), Sánchez-Rodríguez et al. (2021) and Ivanović et al. (2021), giving population a close idea about the products, dose and timing for Zn treatments. However, all the experiments were developed in small-sized plots; according to Jones et al. (2021) results in small-sized plots are not always in accordance with results in big plot experiments or "*real life*", so the present study aims to determine the effectiveness of a proposed Zn enriching program in wheat crop under Mediterranean conditions in half-hectare-sized plots.

MATERIALS AND METHODS

Site of the field experiment

Field experiments were conducted in Elvas, southern Iberian Peninsula (38º 89' N, 7º 05' W, 326 m a.s.l.), Portugal, in a Luvisol under rainfed Mediterranean conditions. Weather-related parameters for this area are as follows: average annual rainfall, 291.5 mm: 40.9 % of the total annual rainfall September–November (Autumn); 13.7 %, December–February (Winter); 42.9 %, March–May (Spring) and 2.4 %, June-August (Summer); average of minimum temperature in the coldest month (January), 3.4 ºC and average of maximum temperature in the warmest month, 30 ºC, with 12 days with maximum temperatures between 25-30 °C and 15 days with maximum temperatures above 30 °C, after 9th.

It is very important to highlight that on the studied year an excessive autumn rainfall caused a delay in the sowing date until January 5th and secondly, while an important heat wave took place at early May (just in grain filling growth stage).

Experimental design

The experimental designed comprised three big-sized plots of one hectare each where were sowed three different bread wheat varieties. Main plots corresponded to bread wheat varieties Almansor, Paiva and Roxo, and subplots (half hectare) corresponded to treatment (Zn treated or control with 0-Zn treatment). Foliar applications of Zn were done at anthesis (28/04/2022) and milk or milk-dough (depending on the variety – 18/05/2022) stage at a rate each time of 0.25 % (w/v) of $ZnSO_4 + 7H_2O$ with 600 L ha^{-1} (total per treatment of 0.5 % w/v $ZnSO_4 + 7H_2O$). Supplementary irrigation was applied in control plots.

Crop management

Weeds were controlled in pre-emergence with clortoluron + diflufenican (2.5 L ha^{-1}) (Trigonil, Bayer Crop Science S.L.) + glyphosate (1.5 L ha^{-1}) and in post-emergence with iodosulfuron-metil-sodio + mefenpir-dietil + mesosulfuron-metil + tiencarbazon-metil (Atlantis, Bayer Crop Science S.L.) at a rate of 0.4 kg ha^{-1}. Conventional tillage treatment included moldboard plowing and disk harrowing at the beginning of autumn, and/or vibrating tine cultivation to prepare a proper seedbed before sowing. As said before, the bread wheat cultivars were "Almansor", "Paiva" and "Roxo". Experiments were sown in January 5th 2022 at a seeding rate of 140 kg ha$^{–1}$ in paired-row 18/36 wide. N-P-K fertilizer (20-8-10) was applied before sowing at 200 kg ha$^{–1}$ in all the plots. An additional amount of 60 units of N ha^{-1} were applied by means of 27% N product.

Measurements

Soil samples and properties

Before sowing, six representative soil samples (0-30 cm) were taken from the experimental field. Soil samples were air dried and sieved to < 2 mm using a roller mill. Texture was determined gravimetrically showing the soil a loamy texture. Soil pH was determined using a calibrated pHmeter (ratio 10 g soil: 25 ml deionized H_2O) and a pH of 7.1 was obtained. Organic Matter (SOM) of the soil was determined by oxidation by dichromate, giving an average value of 1.4%. A portion of each soil sample was finely ground (< 0.45 mm) using an agate ball mill (Retch PM 400 mill) to determine total Zn in soil and obtaining a mean value of 0.4 ppm.

Grain and leaves samples preparation and analysis

Leaf samples were taken two days before the second Zn application and one week after. Nine flag leaves per replicate (three replicates along the whole plot) were cut and immediately N-frozen in the field. After one week in -80 ºC storage, leaves were freeze-dried (LyoQuest,Telstar®, Azbil Telstar Technologies S.L., Spain) and grinded (Cecotec TitanMill 200, Cecotec Innovaciones S.L., Spain) for further analysis.

For determination of total phenolic (TPC), ortho-phenolic (OP) and flavonoids (Flav.) contents, and DPPH free radical scavenging capacity (RSC), 40 mg of freeze-dried leaf powder were used. Prior to analysis, samples were extracted with 1.5 mL of 70 % methanol: water mixture. After that, orbital shaking for one hour (700 rpm at 25 °C) took place. Then centrifugation during 15 min at 4 °C (10,000 g) was carried out and the supernatant was collected. The process was repeated three more times up to having a 6 mL volume). The extract was stored at -80 °C. Thus, total phenolic content was determined by the Folin-Ciocalteau method following Singleton et al. (1998) with slight modifications. A mixture of 20 μL of leaf extract, 90 μL of distilled water and 10 μL of Folin-Ciocalteau reagent were added to a 96-well microplate and left in the dark at room temperature for 6 min. After that, 80 μL of 7 % sodium carbonate (Na_2CO_3) solution were added to each well and incubated in the dark at room temperature for 2 h. Finally, the absorbance was measured on a plate reader (800TS, BioTek Instruments, USA) at 740 nm. Gallic acid standards from 0 to 1 mg mL^{-1} were analyzed and a standard curve was created to calculate total phenolic content of the samples. Similarly, total ortho-phenols were determined by using a sodium molybdate colorimetric assay adapted from Singleton et al. (1999). In this case, 160 μL of leaf extract were mixed with 40 μL of 5% sodium molybdate solution and added to a 96-well microplate. Plate was left in the dark at room temperature for 15 min and then the absorbance was read on the plate reader at 340 nm. The concentrations of ortho-phenols were calculated thanks to a standard curve (0-1mg mL^{-1}) using gallic acid. In addition, the aluminium chloride colorimetric assay adapted from Chang et al. (2002) and described by (Aldhanhani et al., 2022) was used to determine total flavonoid content. Thus, a mixture of 60 μL of leaf extract and 28 μL of 5% sodium nitrite ($NaNO_2$) solution was added to a 96-well microplate and left in the dark at room temperature for 6 min. After that, 28 μL of 10% aluminium chloride ($AlCl_3$) solution was added to each well and incubated again for 6 min in the dark. Finally, 120 μL of 4% sodium hydroxide (NaOH) solution was added to each well and, after a gentle agitation the absorbance was read on a plate reader at 340 nm. In this case, flavonoid's concentration was calculated using a standard curve (0-0.5 mg mL^{-1}) created with catechin standards. To finish with antioxidants determinations, total antioxidant capacity was measured according Xu and Chang et al. (2007) using the DPPH (2,2-diphenyl-1-picrylhydrazyl) free radical scavenging method. In this case, 22 μL of leaf extract were mixed with 200 μL of 120 μM DPPH solution dissolved in 70% methanol: water and added to a 96-well microplate. Mixture was left in the dark at room temperature for 30 min and the absorbance was measured on a plate reader at 490 nm. Trolox standards from 0 to 1 mg mL^{-1} were used to determine the calibration curve (Albadwawi et al., 2022). Each sample was determined in triplicate for any colorimetric assay.

Wheat harvest took place in June 20th using a 1.5 m wide Nurserymaster Elite Plot Combine (Wintersteiger, Austria) and grain yield was determined. Thousand grain weight (TGW) was obtained using a grain counter (Pfeuffer), while test weight and total N content was determined using near infrared equipment (Foss Infratec Grain Analyzer™ 1241, Foss). Bread making wheat grain was ground with a Laboratory Mill CD1 (Chopin, France) to obtain white flour and small aliquots of grain of each variety were milled with an IKA Labortechnik A10 laboratory mill (IKA®-Werke GmbH & CO. KG, Germany) to obtain their wholemeal flours to be analyzed for the mineral profile. The Alveogram index (W), dough tenacity (P) and extensibility (L) of the wheat flour were determined using a Chopin Alveograph (Model Alveographe RCV4, Chopin, France), according the standard AACC Method (2000). Dry gluten content of the flour was determined using the Glutomatic system (Perten Instruments, Sweden).

Both, milled grain and leaves, were subjected to a XRF analysis to determine their mineral profile. X-Ray fluorescent analysis were carried out by means of an Olympus Vanta XRF Analyzer (Olympus Corporation, Japan) equipped with sensitive large-area silicon drift detectors and 50 kV X-ray tubes with a rhodium (Rh) anode.

Colorimeters and stoma size

Colorimetric parameters both in milled grain and leaves were determined with a scanning spectrophotometric colorimeter (Agrosta, France) equipped with a sensor with 6 phototransistors for specific transmittance regions of the spectrum (red – 670 nm; orange – 600 nm; yellow – 570 nm; green –500 nm; blue – 450 nm and violet – 380 nm).

In addition, the CIELAB reflected colour of the samples (both milled grain and leaves), described by the coordinates L* (darkness/whiteness), a* (greenness/redness) and b* (blueness/yellowness), was measured using a Minolta Colorimeter CR-300 (Minolta Camera Co., Japan) (Nadeem et al., 2022). Samples were measured in triplicate after instrument calibration.

Stoma size was determined by means of a Jeol JSM-T330A SEM Scanning Electron Microscope with Tracor Northern II Series TN-5502N EDS System (Ahmed et al., 2021).

Statistical analyse

The effect of the variety and the zinc treatment and their combination on each test was evaluated by two-way analysis of variance (ANOVA) test when normality (Bera-Jarque test) criteria were satisfied. Tukey test for multiple comparison was used when significant differences ($P<0.05$) were found in the ANOVA. Pearson correlation tests were performed between the different parameters. Principal component analysis (PCA) and discriminant analysis (DA) were conducted on the elemental composition traits for each wheat variety and Zn treatment with the aim of determining the most explanatory variables in the method. All these analyses were performed with the XLStat (Addinsoft, 2022) 'add-on' for Microsoft Excel.

RESULTS

Effects of treatments on grain and leaf mineral components

Zinc content (ppm), as well as Cl, Mn, Rb and S contents, significantly varied with the application of Zn when studying the interaction Zn application * variety ($P<0.05$) (Table 1). However, only in Zn data an influence of Zn treatment in two out of the three varieties was found, while for the rest of the elements, variability was given by the variety and not the Zn application. Thus, as shown in Table 1, Almansor and Roxo bread wheat varieties presented an interesting increase in grain Zn amount (from 48.56 and 57.56 ppm to 73.89 and 79.22 ppm, respectively). Elements such as Ca, Cu, Fe, K, P, Si, Sr and Th, did not show any significant differences in the interaction (Table 1. Supplementary material).

In wheat leaves, the interaction wheat variety * Zn treatment was not significant regarding the amount of Ba, Cu, Mo and Th (Table 2. Supplementary material), however the effects were inconsistent regarding the Zn application in the rest of the mineral elements except Mn, Rb and Zn (Table 2). Manganese amount in Roxo leaves after a Zn application of 0.25 % (w/v) of $ZnSO_4 + 7H_2O$ in anthesis stage, showed an increase of nearly 40%, while Rb amount in Almansor leaves after same application decrease close to 60% (Table 2). In case of Zn in leaves, a clear influence of the Zn treatment was found: the amount of Zn increased from about 30 ppm to more than 100 ppm in all cases (Table 2). A significant positive correlation (r=0.92***) was found in this study between the amount of Zn and the amount of Mn in the wheat leaves.

Mineral elements such a Fe, K, Mn and P, showed a significant effect of the Zn treatment in the interaction variety * Zn treatment after the whole Zn application (two applications in anthesis and milk-dough stages) (Table 3). Thus, in variety Paiva, Fe content in leaves increased after Zn applications 247 ppm, as P decreased from 1648.33 ppm to 1325.89 ppm. For Roxo variety, K

Table 1: Mineral grain content (ppm) and significance as affected by the interaction variety*Zn treatment

Mineral element	Almansor		Paiva		Roxo	
	Control	Zn	Control	Zn	Control	Zn
Cl**	2019.22±67.89^{ab}	2217.78±96.25^{a}	1934.33±150.54^{abc}	1717.22±145.54^{bc}	1769.67±29.17^{bc}	1544.22±123.72^{c}
Mn*	100.11±6.99^{ab}	97.22±10.07^{ab}	90.17±7.10^{b}	108.89±1.67^{ab}	115.44±5.24^{ab}	121.33±5.04^{a}
Rb**	7.44±1.21^{abc}	6.67±0.41^{b}	5.67±0.24^{bc}	4.78±0.76^{c}	11.44±1.52^{a}	9.33±1.65^{ab}
S*	2619.11±86.46^{ab}	2671.22±121.13^{ab}	2290.67±75.22^{b}	2423.44±104.73^{ab}	2785.22±106.41^{a}	2636.89±109.02^{ab}
Zn***	48.56±0.59^{b}	73.89±3.00^{a}	43.50±2.04^{b}	48.22±4.48^{b}	57.56±3.93^{b}	79.22±5.78^{a}

Table 2: Mineral leaf content (ppm) and significance as affected by the interaction variety*Zn treatment after the application of half of the Zn treatment (first application in anthesis)

Mineral element	Almansor		Paiva		Roxo	
	Control	Zn	Control	Zn	Control	Zn
Ca*	12262.56±653.27^{b}	13400.17±290.28^{ab}	14957.89±439.00^{ab}	13280.56±581.03^{ab}	15415.11±1587.77^{ab}	15448.11±174.61^{a}
Cl**	30660.22±3313.77^{abc}	36540.50±1706.93^{a}	33043.11±2626.03^{ab}	25541.44±1982.34^{bcd}	23950.78±1508.98^{cd}	21509.78±103.50^{d}
Fe***	194.11±12.65^{ab}	218.50±7.98^{a}	151.67±1.65^{c}	171.11±10.74^{bc}	206.33±4.64^{ab}	214.33±9.40^{a}
K**	31140.11±1273.36^{abc}	35656.83±1879.13^{ab}	31644.00±2401.54^{ab}	31197.56±1909.76^{abc}	28033.33±413.41^{bc}	24847.67±383.13^{c}
Mn***	106.67±6.04^{d}	134.83±8.05^{cd}	162.78±14.83^{bc}	167.67±4.81^{bc}	194.11±9.57^{b}	267.00±9.59^{a}
P*	1664.00±65.95^{b}	189.00±142.79^{ab}	1926.22±145.03^{ab}	1877.33±167.03^{ab}	2521.44±276.89^{a}	1989.33±39.54^{ab}
Rb*	7.00±0.47^{b}	11.67±0.89^{a}	8.44±0.27^{ab}	9.56±1.36^{ab}	9.22±0.49^{ab}	7.56±0.98^{ab}
S***	4538.22±163.04^{b}	5214.00±258.42^{b}	5241.22±380.96^{b}	5667.22±382.10^{b}	8103.00±720.45^{a}	8837.22±59.54^{a}
Si**	25197.78±2337.03^{b}	22264.17±495.76^{b}	32464.00±1662.89^{a}	29887.11±1647.94^{ab}	28937.67±1941.40^{ab}	26475.67±1451.19^{ab}
Sr*	21.67±0.24^{b}	23.33±0.47^{ab}	23.89±0.95^{ab}	23.33±0.41^{ab}	25.67±2.09^{ab}	28.00±1.55^{a}
Zn***	35.49±1.36^{b}	107.50±2.00^{a}	35.00±2.12^{b}	107.78±2.83^{a}	37.11±0.89^{b}	104.78±3.42^{a}

decreased from 26774.89 ppm to 22726,00 ppm, increasing the Mn content about 20 % (Table 3). Zn treatments increased the Zn content in leaves in any variety, but in a different way: thus, while control treatments showed a Zn amount about 30 ppm, Zn treatments increased Zn leaves content up to 146.33 ppm, 329.56 ppm and 260.56 ppm in Almansor, Paiva and Roxo varieties respectively (Table 3). A significant positive correlation (r=0.70***) was found in this study between the amount of Zn and the amount of Fe in the wheat leaves, while negative correlation was found when correlating Zn and P data (r=0.47*). Elements such as Ba, Cu, Mo and Th were not affected by the complete Zn treatment (Table 3. Supplementary material)

The principal component analysis (PCA) developed for the mineral profile of the wheat grains of the three varieties did not show any concluding explanation about the influence of minerals in varieties or zinc treatments, even when it explained more than 75% of the variability of the data (Fig. 1. Supplementary material). However, minerals such as Fe, Rb and Zn, were the most important minerals to explain samples distribution in the PCA of the leaves data, explaining 65.01% of the variability for the first zinc treatment (half of the Zn amount). Thus, these minerals in the leaves after the first part of the zinc treatment clearly separated the samples in axis F2 for the Almansor variety, placing control samples in the down part of the biplot, while zinc-treated samples remained in the upper part (Fig. 1 left). In the same way, Ca, Cl and Sr seem to be the more important minerals to discriminate Paiva leaves samples regarding the zinc treatment. With close to 84% of the variability explained by the two axis of the biplot, Paiva samples after zinc treatment were placed in the right part of the axis F1, while control samples of Paiva remained on the left (Fig. 1 right).

Effects of zinc treatments on grain quality traits

Quality traits were obtained to determine the possible influence of the Zn amount in the protein and rheological properties of the wheat grain and flour, as well as grain colour. Thus, significant differences were found for grain protein content, test weight, TGW, and dry gluten and tenacity of the flour, while grain colour, extensibility and Alveogram index of the flour were not affected by the Zn treatments.

Table 3: Mineral leaf content (ppm) and significance as affected by the interaction variety*Zn treatment after the application of the whole Zn treatment (two applications in anthesis and milk-dough stages).

Mineral element	Almansor		Paiva		Roxo	
	Control	Zn	Control	Zn	Control	Zn
Ca***	13463.78±59.94[c]	12922.78±355.97[c]	15502.33±498.43[b]	16066.11±409.28[b]	18179.89±404.31[a]	18273.56±387.25[a]
Cl***	43801.78±1100.86[a]	41420.33±652.52[a]	32755.00±1347.65[b]	31009.22±1015.04[b]	25000.78±650.09[c]	22759.00±610.67[c]
Fe***	275.33±8.96[b]	274.78±18.21[b]	223.67±4.58[c]	470.67±31.09[a]	247.33±3.06[b]	241.67±5.27[bc]
K***	32419.78±643.85[a]	34056.44±734.38[a]	33584.67±1290.68[a]	31710.67±631.49[a]	26774.89±963.83[b]	22726.00±531.87[c]
Mn***	141.73±7.31[d]	120.00±4.50[d]	163.67±7.48[cd]	202.56±13.55[c]	265.89±13.03[b]	321.67±14.74[a]
P***	1936.67±42.10[ab]	2119.33±91.49[a]	1648.33±112.42[bc]	1325.89±48.31[d]	1504.56±116.42[cd]	1241.44±21.12[d]
Rb**	9.00±0.24[b]	9.56±0.59[ab]	10.83±0.85[ab]	11.67±0.24[a]	10.00±0.24[ab]	8.44±0.68[b]
S***	5337.11±50.48[b]	5573.22±143.34[b]	5404.00±214.72[b]	5271.11±178.34[b]	8394.44±259.80[a]	8731.33±223.22[a]
Si**	32898.56±1108.00[bc]	28241.44±2413.62[c]	34393.67±2161.26[abc]	39398.00±1089.37[ab]	37542.78±3432.17[ab]	43949.11±1391.84[a]
Sr***	24.67±0.41[c]	23.67±1.08[c]	26.67±0.95[bc]	29.67±0.62[ab]	32.78±0.27[a]	33.00±1.22[a]
Zn***	31.56±1.34[d]	146.33±7.97[c]	30.50±2.19[d]	329.56±15.11[a]	31.78±1.09[d]	260.56±7.30[b]

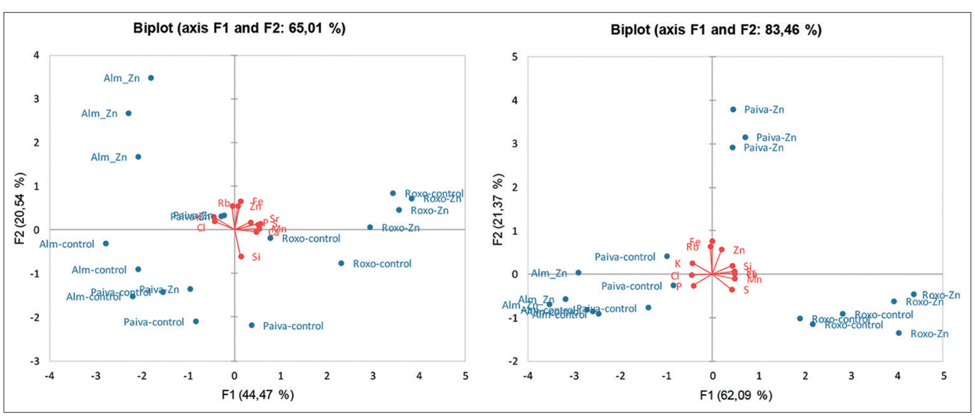

Fig 1. Principal Component Analysis (PCA) biplot of wheat variety-zinc treatment and analyzed minerals in flag leaves after first zinc treatment (left) and second zinc treatment (right).

Table 4 shows how the protein content decrease with the zinc treatments for Almansor variety, while for Paiva and Roxo, protein content increases significantly after zinc treatment. However, even when statistically significant, de difference in percentage of protein is less than 1% in any case. Only Paiva variety showed a higher test weight in its grains after zinc treatment increasing the value from 77.30±0.62 to 79.20±0.14. In the same way, only Almansor exhibited statistical differences in TGW and P when zinc treatment was applied; thus, after zinc treatment, TGW was about 2 g higher than the control treatment TGW, and dough tenacity increases from 98.10±1.68 to 109.17±4.12 (Table 4). Regarding dry gluten, even when statistical analysis revealed some differences for this parameter in Paiva and Roxo varieties, the differences were less than 1% in both cases, increasing dry gluten in Roxo after zinc treatment and decreasing in Paiva. No interesting influence of the zinc treatment in the grain colour was obtained after the analysis (Table 4. Supplementary material).

The principal component analysis (PCA) developed for the quality traits of the wheat grain and flour of the three varieties explained close to 80% of the variability of the data, being protein content and test weight the parameters that better define Roxo and Almansor varieties, while L and W, fit better to explain Paiva variability (Fig. 2).

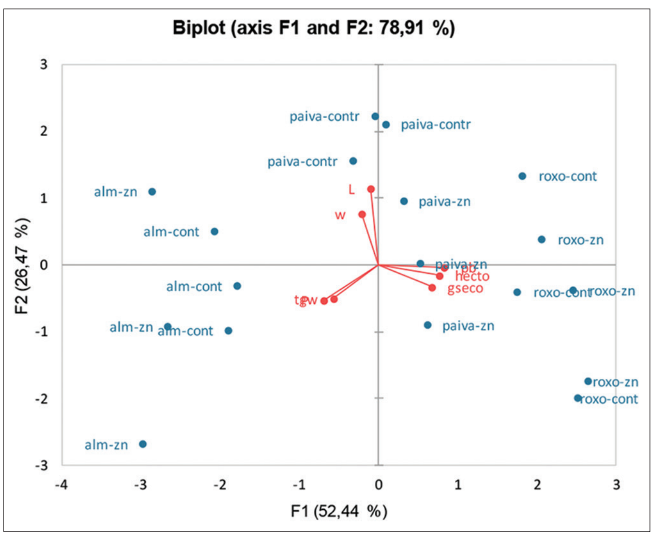

Fig 2. Principal Component Analysis (PCA) biplot of wheat variety-zinc treatment and analyzed quality traits in grain and flour.

Effects of zinc treatments on leaves colour, antioxidants and stoma size.

Little influence of the zinc treatment was found in leaves colour after the analysis: according to the results, wheat leaves seem to be slightly brighter after the first zinc treatment (with the half of the total amount) when comparing with the control leaves (Table 4. Supplementary material), but differences disappear after the whole Zn treatment.

Regarding the antioxidants in leaves, results show (Table 5) that these parameters are very dependent of the cultivar in study; thus, while Roxo variety is barely affected by zinc treatment in regard of OP, RSC, flavonoids and TPC, Paiva wheat variety is negatively affected, decreasing OP content and RSC in both leave sampling times. On the contrary, zinc treatment significantly increases OP content (more than 1000 mg gallic acid (GA) mL^{-1}) and RSC (more than two times) after the half of the treatment and more than three times after the complete zinc treatment in Almansor cultivar leaves (Table 5).

With reference to the stomata size, the cultivars' behavior in regard to the zinc treatment after the complete plant cycle was completely different; while Almansor did not show to be affected by zinc (stomata size in the range of 36-40 microns), Roxo stomatas reduced in more than 10 microns their size after the zinc treatment (from 46 microns in control treatment to 35 microns), and Paiva showed an increase in the stomata size from 30 microns in the control treatment to 47 microns after zinc treatment.

DISCUSSION

According to Szerement et al. (2022), the reaction of the species and/or cultivars to the application of fertilizers enriched with Zn, Se, or Fe differs enormously, both in the amount of element accumulated in the edible parts of the plant and the antagonistic effect between the elements in

Table 4: Quality traits of grain and flour (protein-%, test weight-Kg Hl^{-1}, TGW-g, dry gluten-%, P-mm, L-mm, W-J·10-4) and significance as affected by the interaction variety*Zn treatment.

Mineral element	Almansor		Paiva		Roxo	
	Control	Zn	Control	Zn	Control	Zn
Protein***	14.60±0.07^{e}	14.23±0.04f	16.00±0.15^{d}	16.40±0.12^{c}	17.80±0.01^{b}	18.50±0.14^{a}
Test weight***	77.53±0.08^{c}	77.07±0.29^{c}	77.30±0.62^{c}	79.20±0.14^{b}	80.47±0.04^{a}	80.47±0.15^{a}
TGW***	33.30±0.12^{b}	35.23±0.41^{a}	26.15±0.64^{e}	27.27±1.87^{de}	30.17±0.36^{c}	28.70±0.32^{cd}
Dry gluten***	12.00±0.08^{cd}	12.00±0.10^{cd}	12.18±0.09^{c}	11.88±0.04^{d}	13.38±0.07^{b}	13.65±0.18^{a}
P***	98.10±1.68^{b}	109.17±4.12^{a}	80.90±2.85^{c}	83.55±3.34^{c}	83.58±3.01^{c}	82.78±2.54^{c}
L	115.80±10.47	104.87±24.71	147.50±10.11	107.87±13.87	96.17±21.41	100.10±13.79
W	366.33±18.58	382.50±43.71	375.50±4.91	333.50±22.78	331.83±51.86	362.67±31.10

Table 5: Antioxidants in leaves (ortho-phenols (OP)- mg gallic acid (GA) mL^{-1}; radical scavenging capacity (RSC)- mg trolox mL^{-1}; flavonoids (Flav)- mg catechin mL^{-1}; total polyphenol content (TPC)- mg gallic acid mL^{-1}) and significance as affected by the interaction wheat variety*Zn treatment.

	Control	OP	RSC	Flav.	TPC
FLAG LEAF 1st sample					
Almansor	Control	51141.79±3583.06^{b}	5.13±0.01^{b}	20.06±1.29^{ab}	19.87±4.50
	Zn	65633.03±1407.17^{a}	16.24±1.70^{a}	28.98±3.84^{a}	18.81±5.14
Paiva	Control	63673.40±2696.74^{a}	14.77±3.29^{a}	25.30±5.88^{ab}	21.85±3.46
	Zn	55125.77±2161.20^{b}	7.56±2.10^{b}	18.62±0.13^{b}	17.09±1.41
Roxo	Control	58221.88±1196.48^{ab}	5.96±1.01^{b}	16.09±3.43^{b}	22.15±2.80
	Zn	64973.19±3292.93^{a}	15.25±1.21^{a}	23.22±1.13^{ab}	23.30±2.34
FLAG LEAF 2nd sample					
Almansor	Control	58676.12±3904.07^{bc}	9.41±1.97^{b}	20.25±3.55^{ab}	19.81±2.04^{c}
	Zn	69351.39±1926.97^{a}	19.52±2.42^{a}	22.16±4.33^{ab}	30.57±2.92^{a}
Paiva	Control	66229.21±3429.75^{ab}	17.51±1.17^{a}	25.40±1.19^{a}	24.93±1.66^{abc}
	Zn	52203.86±2666.20^{c}	4.78±0.96^{c}	14.04±0.32^{b}	18.65±0.60^{c}
Roxo	Control	66764.13±2755.94^{ab}	3.68±1.06^{c}	22.82±2.01^{ab}	26.80±3.26^{ab}
	Zn	68071.57±5208.43^{a}	11.18±1.08^{b}	27.47±5.73^{a}	23.59±1.07^{bc}

the uptake process. Thus, Sánchez-Rodríguez et al. (2021) in their study using durum and bread wheat found significant differences between both species, but also regarding the time of application and the fertilizer type. In this case, some treatments did not show any increment in the grain Zn content. Similarly, Luís et al. (2021), using two of the same varieties sown in the present study, found how Paiva variety (which in the present study did not show any increment in the grain Zn content after the treatments), when Zn application occurred in early stages, showed an interesting increase in the grain Zn content. No wonder, then, the different behavior of the three cultivars used in this study, increasing the grain Zn content after the treatment in two cultivars (Almansor and Roxo) but not showing any variation in the third cultivar (Paiva).

However, many times wheat varieties have been shown an increment in the grain Zn content after Zn enrichment programms. In Mediterranean climates or similar, Cakmak et al. (2010), increased up to 7 ppm of Zn in the bread wheat grain doing the applications either in booting+anthesis+milk stage or stem elongation+booting+milk stage+dough stage using 4 kg Zn ha^{-1}, while the previously cited work of Sánchez-Rodríguez et al. (2021), got an increment in Zn grain content after Zn application of about 12 ppm above the control wheat using only 1.28 kg Zn ha^{-1} in foliar spray during flowering. In our case, using less than 0.7 kg Zn ha^{-1}, we obtained an increment of the grain Zn content of 25 and 22 ppm for Almansor and Roxo cultivars respectively, being our treatments in later stages in the cycle: anthesis and milk stage. All these results reveal the great importance of finding the best time for Zn application for each cultivar due to the different responses to the treatments depending on the crop management. This is strongly supported by the fact of having Luís et al. (2021) results, who, working with Paiva variety, the one no significantly affected in grain Zn accumulation after Zn application in our study, reported a great increase in grain Zn content after Zn application in higher doses and earlier growth stages.

Regarding the influence of the Zn treatments on the rest of the mineral in the wheat plant, it was expected the P decrease reported in this experiment, due to the interference of P and Zn at level plant metabolism involving uptake, translocation and utilization (Haldar and Mandal, 1981) and the high correlation between the two elements (r=0.70***). In the same way, the increase of Mn and Fe after Zn treatments was also expected due to the increase in the translocation of Mn from soil to plant tops when the availability of Zn increases (Foy et al., 1978) and the reported positive correlation between Fe and Zn (Mohan et al. 2022).

When analyzing the results of the PCA in this work, minerals that better describe Almansor cultivar, one of the positively affected by Zn treatments, was Fe, Rb and Zn, which can support all the stated above about the relationship between minerals in the plant. In addition, Paiva cultivar, the one not showing influence of the Zn treatments, the minerals that better identify the cultivar were Ca, Cl and Sr; elements also barely affected by Zn treatments.

Quality traits of the wheat in this experiment were poorly affected. Thus, Alveograph parameters stayed the same in most of the cases with or without Zn treatments; only P was slightly increased by Zn in Almansor cultivar. This could be explained by the genetic of the modern wheat varieties: rheological properties are much more dependent of the amount of high molecular weight glutenins (determined by allelic combinations) than of the environment and the crop techniques (de la O Olán et al., 2010). Regarding

TGW, Almansor cultivar showed an increase of about 2 g, which could be expected due to the involvement of the Zn in the enzyme activation, chlorophyll formation, nitrogen metabolism, soluble sugars storage capacity and starch utilization, that resulted in heavier grains due to a higher accumulation of the assimilate in the grains (Singh et al, 1992; Marschner, 1995; Harris et al., 2007; Potarzycki and Grzebisz). These results are supported by authors such as Harris et al. (2007) and Karim et al. (2012), who also found increases of TGW with Zn increases in the wheat plant, due mainly to the influence of Zn. However, Paiva and Roxo cultivars, did not show any influence in TGW, due, probably to the different response of these cultivars to the time of application (Harris et al., 2007). Dry gluten or crude protein of the flour and grain respectively, were also statistically affected by the Zn treatments, nevertheless, the results by cultivar were quite inconsistent: while Almansor did not change dry gluten content, Paiva's one decreased and Roxo's increased. Protein content was negatively affected in Almansor cultivar, but positively in the two other cultivars. All these results should be considered carefully because, even when statistical differences were reported, the numerical differences were really small, less than 1% in any case, minimizing, in a practical way, the importance of these differences. Observing the quality traits globally, it was expected not to find such big differences in protein content because rheological properties barely varied in this study, and proteins and their composition have a great influence in the rheological properties of dough (Peck et al., 2008). In addition, even when it was previously reported that environmental conditions and cultural applications influence protein content, it is also known that genotypic variation also show differences regarding the behaviour of plants facing different crop management (Panozzo and Eagles, 200; Akgun et al., 2016); thus, Akgun et al. (2016) found important differences between genotypes in bread wheat regarding the Zn, P and protein variation under Zn applications.

It is widely known that abiotic stress (i.e. water stress) induced the oxidative damage due to the overproduction of reactive oxygen species (ROS) (Hasanuzzaman et al., 2020), and that plants have evolved physiological and antioxidant defensive mechanisms to combat the toxicity of ROS. Thereby, one of the most common antioxidant generated by plants to defend themselves against ROS is the internal production of superoxide dismutase (SOD) (Sarker and Oba, 2018, Khalil et al., 2022) among others. In our study, leaves samples were taken at the end of the wheat cycle, in May and June when, being in a Mediterranean climate, water stress is quite common. In this regard, Almansor cultivar showed a significant higher amount of antioxidant compounds such as ortho-phenols and also a higher RSC, while Paiva cultivar showed the lowest data for these parameters. This could be explained by the higher capacity of Almansor to metabolize the extra amount of Zn applied with the treatments. In this way, minerals as Zn, Fe and Mn (all them increased in Almansor after Zn biofortification), are known to be cofactors of SOD enzymes, with are associated with ROS scavenging (Assunção, 2022).

Finally, with reference to the stomata size, the three cultivars showed differences in relation to the Zn treatments. So, while Almansor did not show any differences in the stomata size comparing both treated and untreated plants, Roxo cultivar showed a decrease in the stomata size when Zn enrichment took place. On the contrary, Paiva cultivar showed how the stomata size was higher after Zn treatment. In this regard, Zn it is known to be involved in the stomatal regulation (Sharma et al., 1995), and previous studies have reported that Zn application under drought conditions increased the aperture stomata, chlorophyll b and chlorophyll a/b ratio (Sakal et al., 2018). In our study, not modification in the green colour was found, and each variety showed a different response to the Zn treatments regarding the stomata size. We could infer that, derived from the higher size of the stomata in Paiva plants, and the consequent higher evapotranspiration, we could infer that the plant of this cultivars were more stress consuming higher amount of ortho-phenols and showing lower RSC due to the higher activity.

CONCLUSION

In general, Zn application at late stages in the cycle, in farmer's big field, showed a positive effect regarding the Zn increase in grain, in many cases, and in Zn-shoot amount and leaf antioxidants; both interesting to be considered as enriched animal feed. However, genetic diversity of bread wheat cultivars and the most indicated time of application by cultivar, should be taken into account to maximize the positive effects of Zn enrichment. Finally, Zn application did not show great effects in the final quality of the grain in bread wheat.

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AUTHORS' CONTRIBUTIONS

SR, FJL, and ARC conceived and designed the study. SR conducted data gathering and performed statistical analysis.

All authors contributed to the writing of the article and scientific revision.

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The authors have not declared any conflict of interests.

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