Article

Long-Term Zinc Fertilization in Calcareous Soils Improves Wheat (*Triticum aestivum* L.) Productivity and Soil Zinc Status in the Rice–Wheat Cropping System

Pepakayala Vara Lakshmi [1], Santosh Kumar Singh [1], Biswajit Pramanick [2], Mukesh Kumar [1], Ranjan Laik [1], Aradhna Kumari [3], Arvind K. Shukla [4], Arafat Abdel Hamed Abdel Latef [5], Omar M. Ali [6],*
and Akbar Hossain [7],*

1   Department of Soil Science, Dr. Rajendra Prasad Central Agricultural University, Pusa 848125, Bihar, India;
    varam6195@gmail.com (P.V.L.); santoshraupusa@gmail.com (S.K.S.); mukeshsoil@rpcau.ac.in (M.K.);
    rlaik2016@gmail.com (R.L.)
2   Department of Agronomy, Dr. Rajendra Prasad Central Agricultural University, Pusa 848125, Bihar, India;
    biswajit@rpcau.ac.in
3   College of Agriculture, Ganj Basoda, Vidisha 464221, Madhya Pradesh, India; merymitu@gmail.com
4   Indian Institute of Soil Science, Bhopal 462038, India; arvind.shukla@icar.gov.in
5   Botany and Microbiology Department, Faculty of Science, South Valley University, Qena 83523, Egypt;
    moawad76@gmail.com
6   Department of Chemistry, Turabah University College, Turabah Branch, Taif University, P.O. Box 11099,
    Taif 21944, Saudi Arabia
7   Department of Agronomy, Bangladesh Wheat and Maize Research Institute, Dinajpur 5200, Bangladesh
*   Correspondence: om.ali@tu.edu.sa (O.M.A.); akbarhossainwrc@gmail.com (A.H.)

Abstract: Rice–wheat cropping system (RWCS) is considered as the furthermost vital system in the Indo-Gangetic Plains of South-Asia, including India, Pakistan, Bangladesh, and Nepal. Recently, the deficiency of micronutrients like zinc (Zn) has emerged as one of the prime limitations for the sustainability issues of this RWCS in Zn deficient calcareous soils, particularly in India, as a result of the calcareous typic ustifluvents taxonomic nature of the soils. Therefore, a new Zn fertilization approach for soils is very much needed in the intensive RWCS. Thus, a six-year-long investigation was designed with three different modes of Zn application, viz., the application of Zn only in the first year of study, application in alternative years, and application in every year. Four different rates of Zn applications in a hectare of area for a single year, viz., 2.5, 5.0, 7.5, and 10 kg ha^{-1} year^{-1}, and times of Zn application, viz., only at first year, alternative years, and in each year. The major aims of the study were to determine Zn concentration in soil; yield; and Zn accumulation by wheat crop under different application methods of Zn. From this study, it was found that a large portion of the applied Zn was present in the residual fraction, and it was also revealed that increasing the frequency of Zn application resulted in the increment in the crystalline fraction. Significant correlation of water-soluble and exchangeable Zn (WS+EX-Zn), complexed Zn (COM-Zn), amorphous Zn (AMO-Zn), organic Zn (ORG-Zn), total Zn (TOT-Zn), grain yield, and grain Zn uptake by wheat indicated that these Zn fractions were dominant forms in the soil to be utilized by plants under rice–wheat rotation. Concerning yield and Zn uptake by wheat, it was noted that the Zn application at 10 kg ha^{-1} in alternate years was the best Zn application method, while application of Zn at 7.5 kg ha^{-1} in each year also resulted in comparable yield.

Keywords: zinc fertilization; calcareous soils; zinc-uptake; wheat grain yield

1. Introduction

Zinc is a significant constituent for human beings to increase their immunity as this mineral is engaged in more than 300 enzymes for protein as well as carbohydrate metabolism [1,2]. Thus, adequacy in grain Zn in staple food crops like rice and wheat is one

of the major concerns in human diets. The rice–wheat cropping system covering about 27% cultivable area and more than 70% of total food grain production in India is considered as one of the most important and sustainable systems in the Indian part of Indo-Gangetic Plains (IGP) [3,4]. The scientific management of this cropping system is important for nations' food security. However, the use of this intensive system following similar crop production practices over a period of time has resulted in an increase in yield gaps with the deterioration in overall soil fertility and productivity even after the application of synthetic fertilizer at the required rate. This has been attributed to Zn deficiency [5]. Zn has become the third-most-limiting nutrient after N and P, particularly in soils with high pH. Zn deficiency in Bihar soil is even more alarming. About 80–90% of the tested soil samples have displayed Zn deficiency [6]. Only 0.3–3.5% of yearly applied Zn can be taken by most of the crops; such a phenomenon has resulted in Zn accumulation in the soil [7].

Zn deficiency in calcareous soils is more common as about 90% of this micro-nutrient is either adsorbed on soil colloids or precipitated [8]. Soil pH, organic matter (SOM), and $CaCO_3$ in soils improve Zn fertilizer use efficiency [9]. After soil application, Zn is rapidly adsorbed into the soil colloids, forming chelates, and precipitated. Thus, Zn availability of the crops decreases fast [10]. Owing to all these fast changes, the bioavailability of Zn is intricate. On the other hand, phosphorus fertilization also critically affects the Zn availability of plants in calcareous soils. High P application in calcareous soil may impede Zn absorption by the crops, especially rice and wheat [11]. In soils, Zn can exist in seven discrete pools, viz., water-soluble + exchangeable Zn, carbonate and amorphous oxide bound Zn, organically bound Zn, complexed Zn, and Zn pool and crystalline oxide bound Zn [12]. The behavior of Zn in soils and Zn availability to the plants alters with percent as well as the concentration of each Zn fraction to total-Zn in soil [13]. Notouzi et al. [14] previously stated that the entire Zn fractions were augmented with the fertilizer Zn application in the calcareous soil. The authors also reported that the organically bound Zn is the most dominant amongst the different Zn pools. Most of the previous studies focused on the modifications in soil Zn fractions with Zn fertilization for a single crop cycle. Hence, there is a need to know about the transformations of Zn fractions with different Zn loads and frequency over long-term crop-growing seasons on Zn fractions in soil and the availability of Zn for crop uptake.

The proliferation of root systems is decreased owing to having Zn deficiency in many plants [15]. Such phenomenon may be responsible for poor uptake of water as well as essential nutrients from soil resulting in poor growth vis-à-vis yield. Epstein and Bloom [16] reported that not only root development but also flowering as well as fruit setting were significantly poor with severe Zn deficiency in crops. Average Zn concentration ranges from 20 to 35 mg kg^{-1} in grains of wheat in most of the Asian nations like China, India etc. [17]. Tisdale et al. [18] stated that the deficiencies of Zn in a plant usually occur when the Zn concentration is <20 ppm in leaf, and toxicities happen when the values of Zn level in leaf are more than 400 ppm. Hence, the application of Zn in the right dose and frequency is needed for optimum yield without deteriorating soil productivity. However, owing to having a huge research gap in the exploration of suitable Zn application methodology in Zn-deficient upland calcareous soils, long-term study on Zn fertilization is urgently needed to make the farmers aware of suitable Zn fertilization concerning both rate of application as well as the method of application. It is also very much needful to understand the Zn pools in native soil after long-term Zn application. The hypothesis in the current study was to increase the Zn application frequency long term in a calcareous soil to improve soil Zn status and wheat productivity. The current study was carried out with the focal objectives to regulate (i) different fractions of Zn in the soil, (ii) wheat yield, and (iii) Zn uptake by wheat in response to the application of variable Zn frequency and its rates under calcareous soils.

2. Materials and Methods

2.1. Study Site

A six-year-long field research (2012–13 to 2017–18) was initiated during 2012 with a rice–wheat cropping system (RWCS) at the research farm of Dr. RPCAU, Pusa, Bihar, India (25°94′ N, 85°67′ E, and 52 m above MSL). The climate is subtropical humid with hot-humid months of June–September and cold months of November–February with mean rainfall of about 1300 mm annum^{-1}. Frequent floods and droughts are common in this region. The soil is a calcareous typic ustifluvents (as per USDA soil taxonomic classification), sandy loam, alkaline, and is medium in organic carbon, available nitrogen (N), phosphorus (P), and potassium (K) and deficient in sulfur and Zn (Table 1).

Table 1. Initial soil (0–15 cm) physical and chemical properties of the experimental soils.

Particulars	Values	Analysis Methods	Nutrient Level
Texture	Sandy loam (sand 55%; silt 35%; clay 10%)	International Pipette method; Page et al. [19]	-
pH (1:2-soil: water)	8.52	Jackson [20]	-
EC (dS/m) (1:2 soil: water)	0.98	Jackson [20]	-
Organic carbon (%)	0.51	Walkley and Black [21]	Medium
Free $CaCO_3$ (%)	34.3	Black et al. [22]	-
Available N (kg ha^{-1})	246	Subbiah and Asija [23]	Low
Available P (kg ha^{-1})	9.5	Olsen et al. [24]	Low
Available K (kg ha^{-1})	199	Chapman and Prat [25]	Medium
Available S (mg kg^{-1})	12.6	Tabatabai [26]	Medium
Available B (mg kg^{-1})	0.52	Berger and Trough [27]	Medium
Available Zn (mg kg^{-1})	0.67	Lindsay and Norvell [28]	Medium
Available Cu (mg kg^{-1})	2.47	Lindsay and Norvell [28]	High
Available Fe (mg kg^{-1})	16.3	Lindsay and Norvell [28]	High
Available Mn (mg kg^{-1})	4.65	Lindsay and Norvell [28]	Medium
Interpretation	As per values of initial soil (0–15 cm) physical and chemical properties, organic matter (%), K, S, B, Zn and Mn were found in medium level, available N and P were low, and Cu and Fe were in higher level [29].		

2.2. Experimental Treatments and Design

Thirteen treatments such as T_1: Zn at 2.5 kg ha^{-1} in 1st year (in the year 2012); T_2: Zn at 5.0 kg ha^{-1} in 1st year; T_3: Zn at 7.5 kg ha^{-1} in 1st year; T_4: Zn at 10.0 kg ha^{-1} in 1st year; T_5: Zn at 2.5 kg ha^{-1} in alternate years (in the years 2012, 2014, and 2016); T_6: Zn at 5.0 kg ha^{-1} in alternate years; T_7: Zn at 7.5 kg ha^{-1} in alternate years; T_8: Zn at 10.0 kg ha^{-1} in alternate years; T_9: Zn at 2.5 kg ha^{-1} every year (in the years 2012, 2013, 2014, 2015, 2016, and 2017); T_{10}: Zn at 5.0 kg ha^{-1} every year; T_{11}: Zn at 7.5 kg ha^{-1} every year; T_{12}: Zn at 10.0 kg ha^{-1} every year; and T_{13}: Control (No Zn application) were considered for Zn fertilization method and its effect on soil zinc pools and yield of wheat under zinc-deficient calcareous soils. Zn was applied as zinc sulphate ($ZnSO_4 \cdot 7H_2O$), which contains 20% of Zn. Therefore, the application rates of zinc sulphate under Zn at 2.5, 5.0, 7.5, and 10 kg ha^{-1} were 12.5, 25; 37.5, and 50 kg ha^{-1}, respectively. All treatments were organized in a randomized complete block design (RCBD) and repeated three times. The size of each plot was 4 m × 2.5 m.

2.3. Experimental Procedures

Rice (variety *Rajshree*) and wheat (variety HD 2733) were grown in succession. All the experimental plots in each cropping season were applied with 211, 130, and 100 kg ha^{-1} of urea ($CO(NH_2)_2$), diammonium phosphate (($NH_4)_2HPO_4$) (DAP), and potassium chloride (KCl), respectively. The entire level of DAP, KCl, and half of urea were applied to each crop during sowing. Urea was top-dressed at equal split during active tillering, panicle initiation, and flowering stages in case of rice and at crown root initiation and the ear head

initiation stage in case of wheat. Basal application of Zn as zinc sulphate ($ZnSO_4 \cdot 7H_2O$) containing 20% of Zn was done in the rainy season to the only rice crop. A granular form of zinc sulfate was mixed with sand before broadcasting in the field as per treatment. Rice seedlings that were 22 days old were transplanted on 15 July at a spacing of 20 cm row to row and 10 cm plant to plant. Rice was harvested at about 110–120 days after transplanting during the study. After harvesting rice, wheat was sown on 25 November at a spacing of 23 cm between two rows. Wheat was harvested at about 120–125 days after sowing.

2.4. Plant Sample Collection and Chemical Analysis

At maturity, the wheat crop was harvested manually from the whole plot of 10 m^2 areas and the reported yield in this manuscript was the yield of wheat after 6th year of study (in 2017–18). The harvested crop was sun-dried for 2 days. Thereafter, the threshed grain and straw were dried under sunlight for 4 days followed by oven drying for 72 h at 65 °C. Afterwards, the weights of grain and straw were recorded at 12% moisture level. The soil samples were collected with a screw soil auger from 0–15 cm depth at five random locations of each plot after the 6th year of study. The collected samples were mixed thoroughly and partitioned to get 500 g of soil samples from thirteen plots each. The soil samples were dried under shade. Composite wheat seed, as well as wheat-straw samples, were assembled from each of the experimental-plot during harvesting time. Chemical 0.1 M HCl was used to wash the collected samples, which were finally cleaned with deionized (DI) water. The additional moisture was eliminated. Afterwards, the cleaned samples were placed into new brown paper bags for air-drying into an oven at a temperature of 70 °C for 48 h. The samples were ground with the help of Willey Mill (Arthur H. Thomas Type) (Make: Star Scientific Instrument, New Delhi, India).

2.5. Zn Nutrient Analysis

Finely grounded plant samples (0.5 g) were kept in a 250 mL conical flask, and 10 mL of di-acid mixture (HNO_3:$HClO_4$:: 9:4) was added and left overnight for pre-digestion. Subsequently, the contents were placed on a rectangular hot plate (Make: Remi, Mumbai, India) and digested in an open vessel system. The content discoloration to whitish marked the endpoint of digestion. The digestion was stopped when about 1 mL of di-acid mixture was left in the 250 mL conical flask. The flask was allowed to cool. After cooling, distilled water was added to each of the 250 mL conical flask and the contents were transferred to 50 mL conical flask with filtration through Whatman No. 42 filter paper with 2–3 rinsing. The final volume was made up to the calibration mark using distilled water. Finally, the zinc content was analyzed using atomic absorption spectrophotometer (Make: PerkinElmer, Waltham, MA, USA; Model: AAnalyst 200) [29].

The uptake of micronutrient Zn (g ha^{-1}) by wheat grain and straw were estimated by multiplying the Zn concentration (mg kg^{-1}) in wheat grain and straw with the yield of wheat grain and straw, respectively. By adding the Zn uptake by wheat grain and straw, the total Zn uptake by the crop was estimated. Zn harvest index was calculated as grain Zn uptake $\times$ total Zn uptake^{-1}.

2.6. Extraction of Soil Zn Fractions

The sequential extraction procedure of Raja and Iyengar [12] was used to determine the various Zn fractions in the soil after the harvest of wheat after completion of a 6-year study. About 5 g of air-dry soil was placed in a 100 mL polycarbonate centrifuge tube, and the required amount of extractants were added serially, followed by shaking of mixtures for 1 h in a mechanical shaker. Then, samples were centrifuged at 2700 rpm using Remi Centrifugal Machine (Remi, Mumbai, India; Model no. R+8c) and the supernatant was used for the determination of different Zn fractions. The residual soil was washed with 20 mL of DI water, and washing water was discarded, leaving the soil for analysis of the next fraction. The chemicals used for extraction of different fractions were as follows:

(a)   Water-soluble and exchangeable zinc (WS+EX-Zn): 20 mL of 1N NH_4OAc at pH adjusted to 7.0.

(b)   Complexed zinc (COM-Zn): 20 mL of 0.05 M Cu acetate ($Cu(OAc)_2$).

(c)   Organically bound zinc (ORG-Zn): 20 mL of 1% sodium pyrophosphate ($Na_4P_2O_4$).

(d)   Carbonate and amorphous oxide bound zinc (AMO-Zn): 20 mL of 0.1 M HCl.

(e)   Crystalline oxide bound zinc (CRY-Zn): 40 mL of 0.3 M sodium-citrate ($C_6H_5Na_3O_7$) and 5 mL of 0.1 M sodium bicarbonate ($NaHCO_3$).

(f)   Total zinc (TOT-Zn): the total zinc content in soil was determined using atomic absorption spectrophotometer in the digest obtained by digesting soil samples on a hot plate with a 3:1 mixture of HCl and HNO_3 [30].

(g)   Residual zinc (RES-Zn): the residual zinc content was determined by subtracting all forms of Zn extracted from the total Zn content.

2.7. Experimental Data Analysis

The recorded data were analyzed statistically as per a one-way analysis of variance (ANOVA) method for RCBD [31]. The significance of diverse sources of variation was tested by error mean square of Fisher Snedecor's 'F' test at probability level 0.05. For comparison of the mean values of different parameters tested in this study, the least significant difference (LSD) test at 5% probability level was performed. The correlations among different forms of Zn, wheat grain yield, and zinc uptake were tested at 5% level of significance. Data analysis and correlation were performed using SPSS 16.0 for Windows (SPSS Inc. Chicago, IL, USA), and the graphs were made with Microsoft Excel (Microsoft Corp., Pullman, WA, USA).

3. Results

3.1. Soil Zn Pools

The concentrations of WS+EX-Zn ranged between 0.112 and 0.296 mg kg^{-1}, COM-Zn ranged between 0.76 to 1.44 mg kg^{-1}, AMO-Zn from 0.30 to 0.46 mg kg^{-1}, ORG-Zn from 1.62 to 2.75 mg kg^{-1}, CRY-Zn from 2.74 to 3.29 mg kg^{-1}, RES-Zn from 24.26 to 26.95 mg kg^{-1}, and TOT-Zn from 29.57 to 34.65 (Table 2).

Table 2. Effect of different doses and frequencies of zinc application on the content of different fractions of zinc (mg kg^{-1}) in soil.

Treatment	WS+EX-Zn	COM-Zn	AMO-Zn	ORG-Zn	CRY-Zn	RES-Zn	TOT-Zn
T_1	0.115 [hi]	0.82 [i]	0.32 [i]	1.74 [h]	2.74 [efg]	24.26 [d]	29.98 [de]
T_2	0.116 [hi]	0.83 [hi]	0.32 [i]	1.79 [h]	2.65 [fg]	24.49 [cd]	30.03 [de]
T_3	0.124 [h]	0.85 [hi]	0.38 [ef]	1.92 [f]	2.75 [efg]	24.70 [cd]	30.70 [de]
T_4	0.188 [f]	0.87 [gh]	0.35 [g]	1.99 [f]	2.79 [def]	24.62 [cd]	30.67 [de]
T_5	0.136 [g]	0.86 [hi]	0.32 [hi]	1.81 [gh]	2.64 [g]	24.94 [bcd]	30.53 [de]
T_6	0.204 [e]	0.91 [fg]	0.39 [e]	1.98 [f]	2.92 [cd]	24.87 [bcd]	31.20 [cd]
T_7	0.220 [d]	0.97 [e]	0.39 [de]	2.01 [ef]	2.81 [de]	25.13 [bcd]	31.47 [cd]
T_8	0.244 [c]	1.13 [d]	0.42 [c]	2.56 [c]	3.05 [bc]	26.00 [ab]	32.70 [bc]
T_9	0.180 [f]	0.92 [ef]	0.37 [f]	1.92 [f]	3.08 [b]	24.65 [cd]	31.14 [cd]
T_{10}	0.252 [bc]	1.26 [c]	0.43 [bc]	2.42 [d]	3.16 [ab]	25.60 [bc]	32.61 [bc]
T_{11}	0.288 [a]	1.38 [b]	0.44 [b]	2.59 [bc]	3.07 [b]	26.07 [ab]	33.28 [ab]
T_{12}	0.296 [a]	1.44 [a]	0.46 [a]	2.75 [a]	3.29 [a]	26.95 [a]	34.65 [a]
T_{13}	0.112 [i]	0.76 [j]	0.30 [j]	1.62 [i]	2.78 [defg]	24.46 [cd]	29.57 [e]
SEm ($\pm$)	0.003	0.02	0.007	0.04	0.05	0.42	0.53
LSD ($p \leq 0.05$)	0.010	0.05	0.02	0.11	0.15	1.26	1.57

In the column, means with the similar letter(s) are not different at 5% probability level. WS+EX-Zn: water-soluble and exchangeable zinc; COM-Zn: complexed zinc; AMO-Zn: carbonate and amorphous oxide bound zinc; ORG-Zn: organically bound zinc; CRY-Zn: crystalline oxide bound zinc; RES-Zn: residual zinc; TOT-Zn: total zinc. Note: T_1 (Zn at 2.5 kg ha^{-1} in 1st year); T_2 (Zn at 5.0 kg ha^{-1} in 1st year); T_3 (Zn at 7.5 kg ha^{-1} in 1st year); T_4 (Zn at 10.0 kg ha^{-1} in 1st year); T_5 (Zn at 2.5 kg ha^{-1} in alternate years); T_6 (Zn at 5.0 kg ha^{-1} in alternate years); T_7 (Zn at 7.5 kg ha^{-1} in alternate years); T_8 (Zn at 10.0 kg ha^{-1} in alternate years); T_9 (Zn at 2.5 kg ha^{-1} every year); T_{10} (Zn at 5.0 kg ha^{-1} every year); T_{11} (Zn at 7.5 kg ha^{-1} every year); T_{12} (Zn at 10.0 kg ha^{-1} every year); T_{13} (Control, No application).

All fractions of Zn in soil were increased with the increasing doses and frequency of zinc application. The means over the doses and frequency of each Zn fraction were in the following order: TOT-Zn > RES-Zn > CRY-Zn > ORG-Zn > COM-Zn > AMO-Zn > WS+EX-Zn. The RES-Zn fraction accounted for most (78–83%) of the total fraction, followed by CRY-Zn (8.06–9.89%), ORG-Zn (5.48–7.94%), COM-Zn (2.57–4.16%), AMO-Zn (1.01–1.33%), and the WS+EX-Zn (0.38–0.87%) fractions (Table 3). The percent distribution decreased with increasing doses and frequency of Zn application from 83% in control to 78% in the case of Zn applied every year at 10 kg ha^{-1} (Table 3).

Table 3. Effect of different doses and frequencies of zinc application on percentage contribution (%) of each zinc fraction to total zinc in soil.

Treatment	WS+EX-Zn	COM-Zn	AMO-Zn	ORG-Zn	CRY-Zn	RES-Zn
T_1	0.38	2.74	1.07	5.79	9.14	81
T_2	0.39	2.76	1.07	5.97	8.83	82
T_3	0.40	2.77	1.24	6.26	8.96	81
T_4	0.61	2.84	1.14	6.50	9.10	80
T_5	0.45	2.82	1.05	5.93	8.06	82
T_6	0.65	2.92	1.25	6.35	9.36	80
T_7	0.70	3.08	1.24	6.40	8.93	80
T_8	0.75	3.46	1.28	7.83	9.33	80
T_9	0.58	2.95	1.19	6.15	9.89	79
T_{10}	0.77	3.86	1.32	7.42	9.69	79
T_{11}	0.87	4.15	1.32	7.78	9.23	78
T_{12}	0.85	4.16	1.33	7.94	9.49	78
T_{13}	0.38	2.57	1.01	5.48	9.40	83

All zinc fractions' abbreviation and treatments details are available in Table 2.

The percent distribution in the remaining all fractions increased with Zn load. The percent concentration of Zn in different pools increased as per different frequencies of Zn application according to the following order, initial year-application < alternate years-application < every year-application (Table 4). The relative increment in Zn concentration in alternate years and every year over the initial year of Zn application was about 48% and 26% for WS+EX-Zn fraction; 15% and 29% for COM-Zn fraction; 11% and 12% for AMO-Zn fraction; 12% and 16% for ORG-Zn fraction; 3% and 12% for CRY-Zn fraction; 2.93 and 2.31 for RES-Zn fraction; and 4% and 5% for TOT-Zn, respectively (Figure 1).

Table 4. Effect of zinc application frequency (mean of four doses) on different soil zinc fractions (mg kg^{-1}).

Frequency of Application	Zinc Fractions (mg kg^{-1})						
	WS+EX-Zn	COM-Zn	AMO-Zn	ORG-Zn	CRY-Zn	RES-Zn	TOT-Zn
Initial year	0.136	0.843	0.343	1.861	2.733	24.518	30.345
Alternate year	0.201	0.968	0.380	2.091	2.810	25.235	31.476
Every year	0.254	1.250	0.425	2.419	3.150	25.818	32.922
Control	0.112	0.760	0.300	1.620	2.780	24.460	29.572

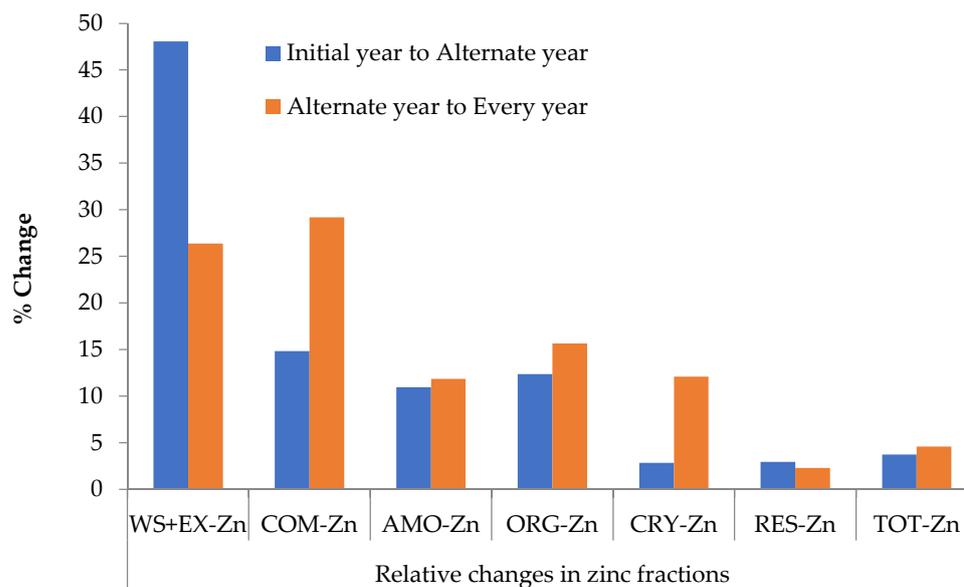All zinc fractions' abbreviations are available in Table 2.

Figure 1. Effect of zinc application frequency on relative changes in different soil zinc fractions (%). All zinc fractions' abbreviations are available in Table 2.

3.2. Wheat Yield

Different Zn fertilization methods significantly influenced the yield of wheat after 6-year of study. Wheat seed, as well as straw yields, were varied from 3.77–4.67 mg ha^{-1} and 6.46–7.97 mg ha^{-1}, correspondingly, due to having different Zn applications (Table 5, Figure 2).

Table 5. Effect of different doses and frequencies of zinc application on wheat yield (mg ha $^{-1}$).

Treatments	Grain Yield (mg ha^{-1})	Straw Yield (mg ha^{-1})	Harvest Index (%)
T_1	3.90 [de]	6.91 [bcd]	36 [a]
T_2	4.07 [cde]	6.46 [d]	39 [a]
T_3	3.94 [cde]	6.72 [cd]	37 [a]
T_4	4.04 [cde]	6.97 [bcd]	37 [a]
T_5	4.12 [cd]	7.35 [abc]	36 [a]
T_6	4.25 [bc]	7.31 [abcd]	37 [a]
T_7	4.56 [ab]	7.90 [a]	37 [a]
T_8	4.67 [a]	7.97 [a]	37 [a]
T_9	4.11 [cd]	7.10 [abcd]	37 [a]
T_{10}	4.59 [a]	7.71 [ab]	37 [a]
T_{11}	4.63 [a]	7.95 [a]	37 [a]
T_{12}	4.18 [cd]	7.29 [abcd]	36 [a]
T_{13}	3.77 [e]	6.46 [d]	37 [a]
SEm ($\pm$)	0.11	0.29	1.2
LSD ($p \leq 0.05$)	0.33	0.88	ns

Values followed by the same letter are not different at 5% probability level. Treatments details are available in Table 2.
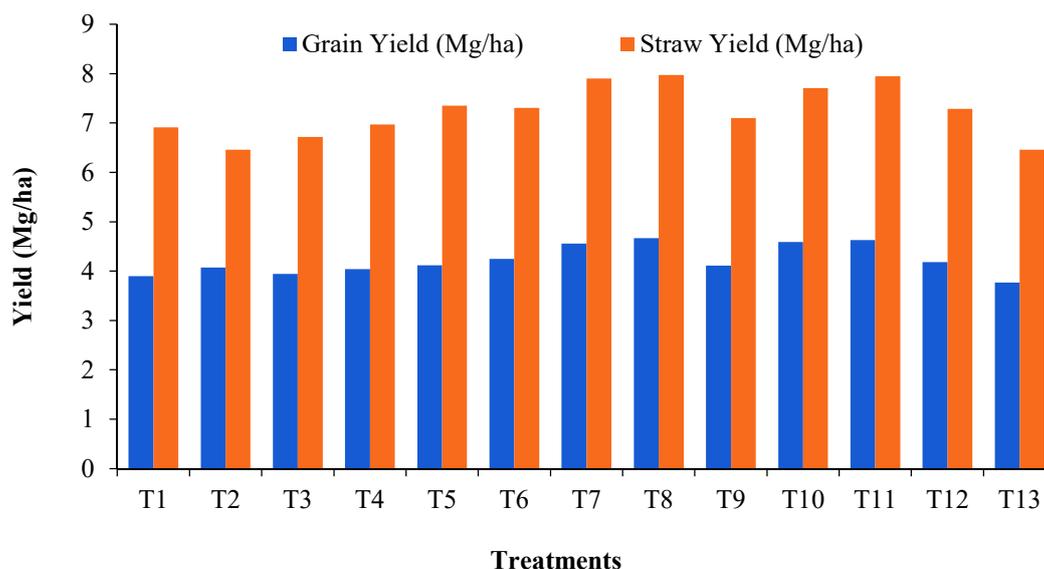
Figure 2. Effect of different treatments on yield of wheat. Treatments details are available in Table 2.

Both grain (4.67 mg ha^{-1}) and straw yield (7.97 mg ha^{-1}) were significantly higher where the crop was applied with 10 kg ha^{-1} Zn in alternate years, and this treatment was statistically at par with the application of Zn at 7.5 kg ha^{-1} in alternate years, Zn at 5 kg ha^{-1} in every year, and Zn at 7.5 kg ha^{-1} in every year. It was also observed that application of Zn at 10 kg ha^{-1} in every year was not capable to boost the wheat grain yield. Conversely, such intensive application of Zn resulted in a reduction in yield to the tune of 9–12% comparing application of Zn at 7.5 kg ha^{-1}–10 kg ha^{-1} in the alternate year or 5 kg ha^{-1}–7.5 kg ha^{-1} in every year. Concerning the harvest index of wheat, different Zn fertilization methods were not found to influence this parameter of wheat significantly (Table 5).

3.3. Zn Uptake by Wheat

The maximum amount of Zn uptake by wheat-grain (115.4 g ha^{-1}) was observed with the application of Zn at 10 kg ha^{-1} in alternate year, and this fertilization method was statistically at par with every year application of Zn either at 7.5 kg ha^{-1} or 5 kg ha^{-1} (Table 6). The straw, as well as total uptake of Zn by wheat plant, also followed the same trend as Zn uptake by wheat-grain. Likewise in case of yield, it was also found that the maximum level of Zn application concerning the highest Zn uptake by plant was 7.5 kg ha^{-1} if applied in every year in the rice–wheat system. However, the level of application can be further increased if applied in an alternate year instead of through yearly application. Thus, an alternate application method can increase the Zn use efficiency in the rice–wheat system. In case of Zn harvest index, no significant differences among different Zn application methods were recorded in the study (Table 6). Zn content (%) in grain and straw of wheat did not vary with different Zn fertilizations (Supplementary Table S1). However, yield of wheat was influenced (Figure 2), which resulted in the variation in Zn uptake by wheat under different treatments.

Table 6. Effect of different doses and frequencies of zinc application on wheat grain, straw, and total zinc uptake.

Treatments	Zn Uptake (g ha^{-1})			Zn Harvest Index
	Grain Uptake	Straw Uptake	Total Uptake	
T_1	96.3 ^{de}	170.7 ^{def}	267.0 ^{cdef}	0.36 ^{a}
T_2	100.5 ^{cde}	159.6 ^{f}	260.1 ^{ef}	0.39 ^{a}
T_3	97.3 ^{cde}	166.0 ^{ef}	263.3 ^{def}	0.37 ^{a}
T_4	99.8 ^{cde}	172.2 ^{def}	272.0 ^{cdef}	0.37 ^{a}
T_5	101.8 ^{cd}	181.6 ^{bcd}	283.3 ^{bcd}	0.36 ^{a}
T_6	105.0 ^{bc}	180.6 ^{bcd}	285.5 ^{bc}	0.37 ^{a}
T_7	112.6 ^{ab}	189.0 ^{abc}	301.7 ^{ab}	0.37 ^{a}
T_8	115.4 ^{a}	196.9 ^{a}	312.2 ^{a}	0.37 ^{a}
T_9	101.5 ^{cd}	175.4 ^{cde}	276.9 ^{cde}	0.37 ^{a}
T_{10}	113.4 ^{a}	190.4 ^{ab}	303.8 ^{ab}	0.37 ^{a}
T_{11}	114.4 ^{a}	196.4 ^{a}	310.7 ^{a}	0.37 ^{a}
T_{12}	103.3 ^{cd}	180.1 ^{bcd}	283.3 ^{bcd}	0.36 ^{a}
T_{13}	93.1 ^{e}	159.6 ^{f}	252.7 ^{f}	0.37 ^{a}
SEm ($\pm$)	2.72	4.51	7.33	0.01
LSD ($p \leq 0.05$)	8.1	13.8	21.9	ns

Values followed by the same letter are not different at 5% probability level. Treatments details are available in Table 2.

3.4. The Relationship among Different Forms of Zn, Wheat Crop Yield and Zinc Uptake

A highly positive and significant relationship between wheat grain yield and WS + Ex-Zn ($r = 0.737$), COM-Zn ($r = 0.688$) fractions of Zn was observed in this study (Table 7). The alike trend of correlation was studied concerning Zn uptake by wheat-grain and different Zn fractions in the soil as well. Correlations were found positively significant regarding all the Zn fractions in soil and grain yield as well as Zn uptake except CRY-Zn and RES-Zn fractions.

Table 7. Correlation among different forms of zinc in soil with wheat grain yield and zinc uptake.

Parameters	WS+EX Zn	COM-Zn	AMO-Zn	ORG-Zn	CRY-Zn	RES-Zn	TOT-Zn	Grain Yield	Zn Uptake
WS+EX Zn	1								
COM-Zn	0.929 **	1							
ORG- Zn	0.932 **	0.910 **	1						
AMO- Zn	0.936 **	0.960 **	0.938 **	1					
CRY- Zn	0.822 **	0.809 **	0.842 **	0.804 **	1				
RES- Zn	0.893 **	0.944 **	0.877 **	0.954 **	0.733 **	1			
TOT- Zn	0.946 **	0.971 **	0.949 **	0.975 **	0.828 **	0.972 **	1		
Grain yield	0.737 **	0.688 **	0.620 *	0.665 *	0.470	0.551	0.609 *	1	
Zn uptake	0.722 **	0.730 **	0.600 *	0.677 *	0.440	0.554	0.636 *	0.925 **	1

* Correlation is significant at the $p = 0.05$ level (two-tailed); ** correlation is significant at the $p = 0.01$ level (two-tailed). All zinc fractions' abbreviations are available in Table 2.

4. Discussion

The majority of zinc in soil (control plot) was presented in RES-Zn form (83.0%), and this form of Zn was followed by CRY-Zn fraction (9.40%) (Table 3). The ORG-Zn, COM-Zn, AMO-Zn, and WS+Ex-Zn fractions were 5.48%, 2.57%, 1.01%, and 0.38%, respectively, as evident from the values of Zn applied plot (Table 3), and the predominance of Zn in the residual fraction has been cited as the main reason for Zn deficiency in Indian soil [32]. With the addition of Zn fertilizer, a large amount of the added Zn was redistributed in the RES-Zn fraction was followed by the CRY-Zn fraction, as evident in this study. This may be more due to high clay and crystalline Fe-oxide content than amorphous oxide content in the soil. In complexed fraction, Zn is weakly bound by humic and fulvic

acids [33]. Due to its weaker bonding with organic ligands, this fraction was readily available to plants and acted as a potential buffering fraction for WS+EX-Zn fraction, whereas in organically-bound fraction, Zn formed stable complexes with insoluble organic matter compounds [34]. The sorption of micro-nutrients in soil depends on soil organic matter (SOM). SOM can form insoluble complexes of micro-nutrients, thus making them immobile. At the same time, SOM can dissolve the trace elements, making them soluble, thereby increasing their availability. Thus, SOM can play dual roles in soil concerning micro-nutrient availability [35,36]. These two fractions respond differently to zinc loading level, pH, and plant availability [37]. The ORG-Zn fraction was at the intermediate level (2.09 mg kg^{-1}). WS+EX-Zn, AMO-Zn, and COM-Zn fractions were very small, possibly due to the conversion of Zn into more stable ORG-Zn and CRY-Zn fraction from less stable, compared to more active COM-Zn and AMO-Zn fraction quickly absorbed into the native soil system. Transformation of Zn fraction is more comprehensive in calcareous soils than the transformations in acid or neutral soils [38]. Organic acids like fulvic and humic acids were solubilized at higher pH levels, which ultimately reduced the COM-Zn fraction, whereas ORG-Zn and CRY-Zn fractions were stable [34]. Higher yield of wheat, as well as Zn uptake by the crop, were found in the application of 10 kg ha^{-1} Zn in alternate years or application of 7.5 kg ha^{-1} Zn every year. This might be due to the fact that the application of Zn at 10 kg ha^{-1} in alternate years or 7.5 kg ha^{-1} in every year resulted in a higher amount of WS+EX-Zn fraction in soil, which is considered as the most available to the plant (Table 2). A higher correlation with grain yield and Zn uptake with WS+EX –Zn (Table 7) also confirms the above statement. A similar kind of observation was previously reported by Arafat et al. [39], Firdous et al. [40] and Zulfiqar et al. [41].

The Zn application usually resulted in the augmentation of the level of each Zn fraction of soil. However, the proportion of all Zn fractions in the soil to total soil-Zn was varied. The percent change in WS+EX-Zn and COM-Zn fractions (which contribute more to plant-available Zn) was more as compared to remaining all fractions. There was relatively little difference was observed in CRY-Zn, RES-Zn, and TOT-Zn fractions, whereas the percent changes variation in ORG-Zn fraction and AMO-Zn fraction were intermediate between the above two states. This may be attributed to the fact that WS+ES and COM-Zn were sensitive to the Zn loading level, but ORG-Zn, RES-Zn, CRY-Zn, and RES-Zn were relatively stable [37]. The percent increase in CRY-Zn fraction with the change in frequency of Zn application from alternate to every year was about 12%, and this was about four times greater than the change in frequency of Zn application from initial to alternate year. However, this CRY-Zn fraction did not contribute to wheat grain yield and Zn uptake. Even though percent change was more in WS+ES and COM-Zn fractions with increased Zn frequencies, their fractional change was minimal. Thus, the major share of applied Zn was distributed in the CRY-Zn fraction. Significant correlation among WS+EX-Zn, COM-Zn, AMO-Zn, ORG-Zn, TOT-Zn, grain yield, and grain Zn uptake by wheat indicated that these fractions of Zn were dominant in the soil to be utilized by plants under the rice–wheat rotation. Raja and Iyenger [11] also observed a highly positive correlation between Zn uptake and Ex-Zn, COM-Zn, and ORG-Zn fraction of Zn.

5. Conclusions

The 6 years of the long study exhibited that a large portion of applied Zn was present in the soil as a residual fraction, and with the increased doses and frequencies of Zn application, the crystalline zinc fractions in the soil were increased. The residual Zn (RES-Zn) fraction accounted for most (78–83%) of the total fraction followed by crystalline oxide bound Zn (8.06–9.89%), organically bound Zn (5.48–7.94%), complexed Zn (2.57–4.16%), carbonate and amorphous oxide bound Zn (1.01–1.33%), and the water-soluble and exchangeable Zn (0.38–0.87%) fractions. The percent concentration of Zn in different pools increased as per different frequencies of Zn application according to the following order, initial year-application < alternate years-application < every year-application. Concerning the wheat-grain yield and uptake of Zn by the crop under different Zn fertilization methods,

it was noticed that Zn application at 10 kg ha^{-1} in alternate years outcome the maximum yield of wheat, and this application method was found at par with Zn application at 7.5 kg ha^{-1} in every year. Although these two Zn application methods were at par, from the 6-year-long study, it was found that the alternate-year application of Zn at 10 kg ha^{-1} accounted for a total 15 kg of lower Zn application as compared to the application of Zn at 7.5 kg ha^{-1} in every year. Thus, an alternate year application method can increase the Zn use efficiency for the wheat crop grown in the rice–wheat system. Hence, from these 6 years of study, it may be stated that the soil application of Zn fertilizer at 10 kg ha^{-1} in alternate years is the most improved Zn fertilization method for Zn deficient calcareous soils.

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