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INFLUENCE OF ZINC FERTILIZATION ON MORPHO-PHYSIOLOGICAL ATTRIBUTES, GROWTH, PRODUCTIVITY AND HEMATIC APPRAISAL OF PADDY RICE

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ABSTRACT

Zinc (Zn) deficiency is one of the main micronutrient disorders in rice and it may inflict its deficiency in the people feeding on it. Selection of rice genotypes with higher potential of Zn uptake is considered as natural and better way to combat malnutrition. Present study evaluated the potential of foliar and soil applied Zn in improving the performance of paddy rice and Zn bioavailability. In glass house, different sources of zinc ($ZnSO_4.7H_2O$ and $ZnCl_2$) and rate treatments for soil (control, 5 kg ha^{-1}, 10 kg ha^{-1}, 15 kg ha^{-1}and 20 kg ha^{-1}of Zn) and foliar application (control, 0.25%, 0.5%, 0.75% and 1% solution) were used. Selected rate and source treatments were appraised in field experiments. The rice grains harvested thereof were fed to albino rats for Zn bioavailability. Results revealed that soil application with 15 kg ha^{-1} and foliar application of 0.25% solution using $ZnSO_4.7H_2O$ improved the early seedling growth of rice genotypes by modulating the agronomic, water related and biochemical attributes. Field experiments confirmed that Zn application through soil and foliar spray improved the performance of rice genotypes by improving growth attributes, chlorophyll pigments, and yield and resulted in Zn enrichment of rice grains in various genotypes. The maximum weekly body weight gain and white blood cells count of albino rats was observed for feeding the kernels of Accession-164 (high Zn) as compared with the minimum for Super Basmati (low Zn) feed. In crux, soil application of $ZnSO_4$ at 15 kg ha^{-1} followed by foliar application of 0.25% $ZnSO_4$ solution at tillering and heading stage not only recorded the highest growth and grain yield, but also showed maximum bioavailable Zn in the grains. Such rice grains with high Zn concentrations were also effective for combating malnutrition in the tested rats.

Key words: Rice, Zinc, Growth, Seedling, Rats.

INTRODUCTION

Globally, the deficiency of zinc (Zn) has appeared as one of the most alarming issue amongst the developing countries (Younas *et al.,* 2015). Today's vital global challenge is better crop production with improved human health that is only possible by growing cereals cultivars having optimum balance uptake of micronutrients particularly Zn or enhancing Zn concentration in cereals which is basic diet of vast population. As for micronutrients deficiency is concerned, Zn deficiency is taking place in crops (cereals, fodders, fruits and vegetables etc.) humans and animals. The main threat to human health and one of the major causes of death considered globally is the Zn deficiency. About 1.1 billion people are at menace of Zn deficiency and ~90% of these people reside in Africa and Asia (Kumssa *et al.,* 2015). Human beings are dependent mainly on plant directly or indirectly for diet. Therefore, whole or part of plant used for food must contain balanced amount of Zn. As Zn is an imperative micronutrient and it has number of essential functions in plant systems.

Zinc is required for the synthesis of tryptophan which is a precursor of auxin and it also acts as a cofactor for more than 300 enzymes (Aravind and Prasad, 2003; Lopez-Millan *et al.,* 2005). It has role in maintaining the integrity of biological membranes, pollen formation, disease resistance, protein synthesis and photosynthesis (Hajiboland and Amirazad, 2010; Sinclair and Krämer, 2012). Globally its deficiency in cultivated soils affects the productivity up to 30% (Alloway, 2008). In Pakistan, approximately 70% of cultivated soils are Zn-deficient (Hamid and Ahmad, 2001; Kumssa *et al.,* 2015). This problem becomes more severe in rice-wheat cropping systems (Mandal and Das, 2013). Agronomic biofortification is economically sustainable and practically adoptable solution to overcome the Zn deficiency issue in rice (Zaman *et al.,* 2017). Micronutrients, including Zn, can be supplied by different methods like seed treatment (priming, coating), soil fertilization and foliar spray (Johnson *et al.,* 2005; Rehman *et al.,* 2017).

Various methods of Zn application may differentially influence yield and grain Zn concentration. Soil Zn application may contribute to better Zn nutrition of human beings (Cakmak, 2008; Zoz *et al.,* 2012; Hussain *et al.,* 2013). However, only high rates ensured optimum Zn concentration in rice grains. The most-used method of Zn fertilizer application is soil application. Zn can be applied to soil through broadcasting, band placement in the vicinity of the seed, or irrigation (fertigation). Selection of appropriate Zn sources for soil application can also act as an alternative strategy to improve plant availability of Zn under lowland conditions. Soil application is a promising strategy for improving Zn concentration in tissues as well as increasing growth and grain yield in rice (Khan *et al.,* 2003; Hussain *et al.,* 2012).

Foliar spray of the fertilizer is an efficient way to correct the deficiency of the particular nutrient. Foliar application of nutrients includes the possibility of nutrients to the plants when soil conditions restrict the root up take, or during rapid growth periods, requirement may exceed root supply. Foliar application can avoid the problems of Zn binding in soil, but the time of Zn application should be around flowering for increasing grain Zn concentration. Similarly, Ebrahim and Aly (2004) reported that the foliar application of Zn sulphate increased the zinc content of leaf and protein content of grain. Foliar application of Zn increases the nutrient concentration in leaves and improves the nutrient balance leading to yield increase (Shaaban, 2001; Esfandiari *et al.,* 2016). However, the positive effects often depend on the concentration of nutrient in the specific crop.

Proper availability at the peak periods of crop demand and the costs involved in Zn fertilization are crucial for harvesting good rice yields and making final recommendations regarding the method of its application under field conditions. Realizing the importance of Zn for plant growth, the severity of its deficiency in soils and plants, and the significance of its bioavailability, an attempt has been made to optimize the most suitable levels and sources for soil and foliar Zn application in rice in controlled conditions. A field appraisal of selected levels and source for soil and foliar Zn application was carried out for enhancing growth, kernel productivity, and Zn biofortification of rice grains. Such Zn-biofortified rice grains were also assessed for Zn bioavailability in albino rats.

MATERIALS AND METHODS

Pot Experiments

Study site and experimental design: Two separate glass house experiments were conducted at Nuclear Institute for Agriculture and Biology, Faisalabad, (31º23′55″ N, 73º2′2″ E) Pakistan during 2015. Experimental treatments were arranged in three factor completely randomized design with three replications. Five Zn efficient genotypes of rice viz., Super Basmati, Accession-126, Accession-154, Accession-164, Accession-175 selected from a screening experiment containing 183 accessions/ genotypes on the basis of grain Zn contents and yield potential (data not shown) were used in this study. Seeds were obtained from the Plant Genetic Resource Institute (PGRI), National Agricultural Research Centre (NARC), Islamabad, Pakistan. The foliar experiment comprised of five treatments viz., control, 0.25%, 0.5%, 0.75% and 1% solution while for soil application control, 5 kg ha^{-1}, 10 kg ha^{-1}, 15 kg ha^{-1} and 20 kg ha^{-1}of Zn applied using two sources of Zn viz., $ZnSO_4.7H_2O$ and $ZnCl_2$.

Crop husbandry: Ten seeds of each genotype were sown in 10 kg soil filled earthen pot (45 cm × 30 cm) on 15th March, 2015 under glass house conditions. The pots were kept under controlled conditions and replicated thrice using completely randomized design (CRD). The maximum and minimum temperatures during the experiments were maintained at 29ºC and 21ºC, respectively, and the relative humidity was about 60%. For foliar application, the nutrient spray from each source was done 30 DAS (Days after sowing), while for soil application, all the Zn was applied at the time of sowing. Basal fertilizer of urea, di-ammonium phosphate and potassium sulphate were applied at the rates of 24 g (N), 40 g (P), and 62 g (K) per pot. The experiment was harvested at 45 DAS on 30th April, 2015.

Data collection: From both experiments, randomly selected three plants were tagged for measuring plant height. After harvesting, the same tagged plants from each pot were counted for number of tillers. For chlorophyll contents, fresh leaves were cut into 0.5 cm segments and extracted overnight with 80% acetone at -10°C. The extract was centrifuged at 14000 rpm for 5 min and absorbance of supernatant was read at 645 and 663 nm using a spectrophotometer (T60 U Spectrophotometer PG Instruments, Limited, USA), the readings were taken according to Nagata and Yamashita (1992). For relative leaf water content (RWC), fresh leaves (0.5 g) were weighed and were floated on water for 4 h, and saturated weight (WS) was measured thereafter. These leaves were dried for 24 h at 85°C to determine dry weight (Wd) and readings were taken by following the method of Barrs and Weatherly (1962). To determine membrane permeability, leaf electrolyte leakage was measured following the protocol of Blum and Ebercon (1981). Six leaf segments of similar size were lightly washed with distilled water and immersed in a test tube having 6 ml distilled water for 12 h at room temperature. Then electrical conductivity (EC_1) of solution was measured with a conductivity meter (Model DDS-11A, Shanghai Leici Instrument Inc., Shanghai, China). Samples were then heated in boiling water for 20 min and cooled to room temperature. The conductivity of killed tissues (EC_2) was again measured. Electrolyte leakage was measured as the ratio of EC_1 to EC_2 and expressed in percentage. For shoot Zn concentration, a di-acid mixture (HNO_3:$HClO_4$ in the ratio of 2:1) method was used by following the protocols of Jones and Case (1990).

Field Experiment

Study site and experimental design: The experiment was conducted at Research Farm of Nuclear Institute for Agriculture & Biology (NIAB), Faisalabad, Pakistan during *kharif*, 2015 and was laid out in randomized complete block design in factorial arrangement of treatments and was replicated thrice.

Same set of genotypes was used as in the pot experiment. There were four Zn treatments (as $ZnSO_4.7H_2O$), non-Zn application as control, soil application (SA) @ 15 kg ha^{-1}, foliar application (FA) @ 0.25% (at tillering and heading) and soil application followed by two foliar applications as in previous treatments.

Crop husbandry: Rice seeds of selected genotypes were sown on 26th June of 2015 using hand drill. Seedlings (30-35 days old) were manually transplanted in puddled fields by maintaining R × R and P × P distance of 22.5 cm. At transplantation, uniform application of fertilizer in the form of 100 N, 67 P and 60 K kg ha^{-1} by applying urea, di- ammonium phosphate and potassium sulphate, respectively. At 25 days after transplanting, the second dose of 60 kg N ha^{-1} was applied. All the Zn by soil application method was applied at the time of sowing while foliar application of Zn was done at tillering and heading stages. During crop growth, to maintain submerged soil conditions canal water was used. Irrigation water was maintained at a depth of 3-4 cm at transplantation and one week afterwards at a depth of 5–6 cm throughout the growing season till one week before harvesting. In total, 19 irrigations (60 acre inches = 6249.64 m^3), were applied to the crop. Meteorological data during the crop season was collected as shown in the Fig. 1. For weed control, Ethoxy sulphuran @ 200 g was applied and hand weeding was also done at specific intervals. Crop was harvested manually on 21st November, 2015.

Data collection: For growth attributes leaf samples were collected at 60 days after transplanting (DAT) and 75 DAT. Total plants in 1 m^2 were harvested from each plot. Immediately after harvesting the fresh weight was recorded. For measuring the dry weight plant a random sample of 100 g was taken from each treatment. For leaf area measurement, 5 g leaves sample was taken and leaf area was measured by leaf area meter (Model: Licor 3000). Maximum leaf area index and crop growth rate was calculated by following the protocols of Watson (1952) and Hunt (1978), respectively. Dry matter production (DMP) per plot was calculated at flowering and harvesting stage and then converted into kg ha^{-1}. Chlorophyll contents and electrolyte leakage were determined on the after 15 days of application of Zn foliar application by following the protocols as described in pot study. Whole plots were harvested and threshed separately and clean rough rice was air-dried. Yield of each plot was weighed and values were adjusted to 12% moisture and expressed in kg ha^{-1}. Grain zinc concentration was determined by following the method of Jones and Case (1990).

Animal feeding Experiment

Study site and experimental design: This experiment was carried out at Institute of Pharmacy, Physiology and Pharmacology in the University of Agriculture, Faisalabad (31º25′52″ N, 73º4′10″ E), Pakistan during December, 2015 and was laid out in CRD.

Experimental treatments: Zinc biofortified rice kernels were produced as described. After harvesting the rice, kernels of all five genotypes was analyzed and grouped into five different categories of grain having different levels of Zn (i.e. 27, 45, 50, 55 and 59 mg/kg dry weight). The kernels of rice genotype (Super Basmati) were considered as control.

Experimental conditions for animals: Forty male Wister strain albino rats having initial body weight of 100 ± 10 g were purchased from same department. The rats were acclimated for five days prior to experiment with an evaluation of their health status. Animals were kept in metallic cages and maintained in an environmentally controlled room 22 ± 2^0C with relative air humidity of 55 ± 5%, and optimum lightening in a daily cycle (i.e. 12 h light/12 h darkness). The experiment was performed according to the rules and regulation protocols accepted by Local Ethical Commission for Investigation on Animals. Rats were maintained in collective cages (4 rats per cage) as treatment plan. Rat feed was made by following AIN standards and during the whole period (one month) of experiment, all rats were supplied with deionized water to eliminate additional Zn sources. After one month feeding of these Zn biofortified kernels to male albino rats their body weight was calculated while for hematic appraisal blood was collected in EDTA-K2 solution tubes for the determination of red blood cells (RBC), white blood cells (WBC), Hemoglobin (HGB), Hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC) using an automated hematology analyzer (Sysmex, Tokyo, Japan).

Statistical analysis: Data from each experiment were analyzed for analysis of variance (ANOVA) using Statistics 8.01 software. Least significant difference (LSD) test at 5% probability level was applied to compare the treatment means. Correlation coefficients among different traits were calculated using a computer program MINITAB 14.

RESULTS

Foliar application (Pot experiment): All foliar application treatments significantly improved agronomic, water related and biochemical attributes. However, foliar application with 0.25% $ZnSO_4$ was more effective than the other treatments in improving the plant height, tillers per plant, relative water contents, chlorophyll contents, shoot zinc contents and decrease of electrolyte leakage (Table 1). Maximum plant height (55.8 cm), tillers (4.4 per plant), RWC (55.9%), chlorophyll *a* (3.6 mg L^{-1}), chlorophyll *b* (3.1 mg L^{-1}), total chlorophyll contents (6.6 mg L^{-1}) and shoot zinc contents (60.4 ppm) and minimum electrolyte leakage (13.4%) were observed and on the other hand foliar application was done with 0.25% Zn using $ZnSO_4$ over control. Foliar application with higher concentrations than 0.25% with $ZnSO_4$ or with $ZnCl_2$ did not improve the agronomic, water related and biochemical attributes further. However, in different genotypes the order for increase in plant height was, Super Basmati > Accession-175 > Accession-164 > Accession-126 > Accession-154, for tillers per plant, Super Basmati > Accession-175 > Accession-126 > Accession-164 > Accession-154, for water related traits, the increase was in order, Super Basmati > Accession-175 > Accession-164 > Accession-126 > Accession-154. Regarding chlorophyll *a* the increase was in order, Accession-175 > Super Basmati > Accession-154 > Accession-126 > Accession-164, for chlorophyll *b,* Accession-164 > Accession-126 > Super Basmati > Accession-175 > Accession-154 and for total chlorophyll contents Accession-175 > Accession-164 > Accession-126 > Super Basmati > Accession-154. While for shoot zinc contents, Accession-164 > Accession-126 > Accession-175 > Accession-154 > Super Basmati, for electrolyte leakage, Accession-154 > Accession-126 > Accession-164 > Accession-175 > Super Basmati.

To substantiate the validity of above findings in different rice genotypes, agronomic, water related and biochemical attributes were correlated with each other (Table 2). Electrolyte leakage correlated negatively with plant height, RWC, shoot zinc contents, tillers per plant and chlorophyll contents. However, total chlorophyll contents and tillers per plant positively correlated with shoot zinc contents, relative water contents and plant height. The shoot zinc content was positively correlated with chlorophyll *b*. Similarly RWC showed positive correlation with tillers per plant and total chlorophyll contents. However, chlorophyll *a* was negatively related to chlorophyll *b* (Table 2). Regarding the interactive effect of different factors like genotypes × levels of Zn, genotypes × zinc sources and genotypes × levels of zinc showed significant effect on the plant height (cm), *CHL* b (mg L^{-1}) and total tillers (per plant), respectively (Fig. 2; a, b, c). Maximum plant height was observed in Super Basmati where foliar application with 0.25% $ZnSO_4$ was done followed by Accession-175 under the same treatment. However, maximum chlorophyll *b* was observed in Accession-164 followed by Accession-126 under the foliar application of 0.25% $ZnSO_4$ while regarding the interactive effect of genotypes × levels of zinc on the tillers per plant showed that maximum tillers per plant were observed in Super Basmati where foliar application with 0.25% $ZnSO_4$ was done followed by Accession-175 under the same treatment. All the other interactive effects showed non-significant behavior regarding agronomic, water related and biochemical attributes.

Soil application (Pot experiment): All soil application treatments significantly improved agronomic, water related and biochemical attributes. However, soil application with 15 kg ha^{-1} using $ZnSO_4$ was more effective than the other treatments in improving the plant height, tillers per plant, relative water contents, chlorophyll contents, shoot zinc contents and decrease in electrolyte leakage (Table 3). Maximum plant height (52.8 cm), tillers (3.9 per plant), RWC (55.6%), chlorophyll a (3.6 mg L^{-1}), chlorophyll b (3.0 mg L^{-1}), total chlorophyll contents (6.6 mg L^{-1}) and shoot zinc contents (60.4 ppm) and minimum electrolyte leakage (13.7%) were observed 15 kg ha^{-1} of Zn was applied using $ZnSO_4$ as source over control. Soil application with higher concentrations than 15 kg ha^{-1} Zn with $ZnSO_4$ or with $ZnCl_2$ did not improve the agronomic, water related and biochemical attributes further. However, for accessions the order for plant height and water related traits the increase was in order Super Basmati > Accession-175 > Accession-164 > Accession-126 > Accession-154. Regarding chlorophyll *a*, the increase was in order, Accession- 175 > Super Basmati > Accession-154 > Accession-126 > Accession-164, for chlorophyll *b*, Accession-164 > Accession-126 > Super Basmati > Accession-175 > Accession-154 and for total chlorophyll contents, Accession-175 > Accession-164 > Accession-126 > Super Basmati > Accession-154. At the same time, the order for increase in shoot zinc contents, Accession-126 > Accession-164 > Accession-175 > Accession-154 > Super Basmati. Regarding electrolyte leakage, Accession-154 > Accession-126 > Accession-164 > Accession-175 > Super Basmati. Tillers per plant showed non significant behavior for accessions under soil application of Zn using different sources. To substantiate the validity of above findings in different rice accessions, agronomic, water related and biochemical attributes were correlated with each other (Table 4). Electrolyte leakage was correlated negatively with plant height, RWC, shoot zinc contents, tillers per plant and total chlorophyll contents. However, total chlorophyll contents and tillers per plant positively correlated with shoot zinc contents, relative water contents and plant height. The shoot zinc content was positively correlated with chlorophyll *b*.

Similarly, RWC showed positive correlation with chlorophyll *a* and plant height. However, chlorophyll *a* was negatively related to chlorophyll *b* (Table 4). Regarding the interactive effect of different factors like genotypes × levels of zinc showed significant effects on the plant height (cm) (Fig. 3). Maximum plant height was observed in Super Basmati where 15 kg ha^{-1} $ZnSO_4$ was used followed by Accession-175 under the same treatment. All the other interactive effects showed non-significant behavior regarding agronomic, water related and biochemical attributes.

Field experiment: Different rice genotypes and zinc application methods significantly affected the growth, biochemical and agronomic attributes. Maximum LAI, CGR, and dry matter production at the flowering and maturity stage was recorded with soil + foliar application of Zn followed by foliar and soil application of Zn. The maximum LAI (4.90), CGR (64.12 g m^{-2} day^{-1}), dry matter production at flowering stage (9670 kg ha^{-1}) and dry matter production at maturity (12780 kg ha^{-1}) was observed in super basmati, while the minimum LAI (4.07), CGR (48.23 g m^{-2} day^{-1}), dry matter production at flowering stage (8960 kg ha^{-1}) and dry matter production at maturity (10780 kg ha^{-1}) were documented in Accession-154. Regarding zinc application methods, maximum LAI (4.59), CGR (61.45 g m^{-2} day^{-1}), dry matter production at flowering stage (9650 kg ha^{-1}) and dry matter production at maturity (11670 kg ha^{-1}) were observed with soil + foliar application of Zn. The interactive effects of different genotypes and zinc application methods for all growth attributes showed non-significant behavior (Table 5).

Zinc application methods and genotypes significantly ($p \leq 0.05$) affected the biochemical and yield attributes of rice (Table 6). However, soil + foliar application was more effective than the other application methods in terms of improved chlorophyll contents, grain yield, grain zinc contents and decreased electrolyte leakage (Table 6). Maximum chlorophyll *a* (3.64 mg L^{-1}), chlorophyll *b* (3.09 mg L^{-1}), grain yield (4280 t ha^{-1}), grain Zn contents (52.61 ppm) and minimum electrolyte leakage (13.06%) were observed in those plots where soil + foliar application was done at 15 kg ha^{-1} and 0.25% solution.

Animal Feeding Experiment: Feed intake of Zn biofortified rice significantly ($p \leq 0.05$) increased body weight of rats in all groups except the last group assessed weekly (Table 7). Maximum increase of body weight as compared to initial body weight regarding 1st, 2nd, 3rd and 4th week, respectively was observed in those rats which were fed with diet containing 55 mg/kg of Zn (Table 6). However, due to high concentration of Zn in rice kernels the body weight of last group did not significantly improve as compared to rest of groups. The increase in the body weight of male Wister rats was in order $G_4 > G_3 > G_5 > G_2 > G_1$ during whole study. The effect of feed intake of Zn biofortified rice on the hematological parameters of albino rats are presented in (Table 8). Feed intake of Zn biofortified rice diet did not significantly ($p \leq 0.05$) increase the hematological parameters except white blood cells (WBC). Maximum increase (26%) in WBC was observed in the group feed with diet containing 55 mg/kg of Zn relative to control. The minimum increase (14.55%) in the WBC was observed in the group fed with diet containing 45 mg/kg of Zn relative to control.

Table 1. Influence of foliar application with different sources of zinc on agronomic, water and biochemical attributes of rice genotypes in pot experiment

Factor	**Agronomic Trait**		**Water Related Trait**	**Biochemical Attribute**				
	PH (cm)	**T** (per plant)	**RWC** (%)	***CHL* a** (mg L^{-1})	***CHL* b** (mg L^{-1})	**T *CHL*** (mg L^{-1})	**S Zn** (ppm)	**EL** (%)
Genotype (G)								
G_1 (Super Basmati)	53.01^{A}	3.56^{A}	55.64^{A}	3.23^{B}	1.77^{C}	5.00^{D}	42.33^{D}	16.17^{D}
G_2 (Accession-126)	44.35^{C}	2.90^{C}	45.28^{C}	3.05^{C}	4.09^{B}	7.14^{C}	60.33^{A}	19.94^{B}
G_3 (Accession-154)	43.56^{C}	2.66^{C}	44.52^{C}	3.18^{B}	0.62^{E}	3.81^{E}	51.39^{C}	21.28^{A}
G_4 (Accession-164)	48.21^{B}	2.86^{C}	50.75^{B}	0.41^{D}	7.25^{A}	7.65^{B}	61.74^{A}	18.91^{BC}
G_5 (Accession-175)	52.30^{A}	3.20^{B}	52.11^{B}	7.08^{A}	0.96^{D}	8.05^{A}	57.07^{B}	18.64^{C}
Source (S)								
S_1	50.00^{A}	3.26^{A}	50.67^{A}	3.43^{A}	2.98^{A}	6.42^{A}	55.51^{A}	18.54^{B}
S_2	46.56^{B}	2.81^{B}	48.65^{B}	3.35^{B}	2.89^{B}	6.24^{B}	53.63^{B}	19.45^{A}
Level (L)								
Control	39.03^{E}	1.90^{E}	44.33^{E}	3.21^{E}	2.84^{E}	6.05^{E}	48.53^{E}	24.46^{A}
FA with 0.25% solution	55.79^{A}	4.40^{A}	55.92^{A}	3.58^{A}	3.05^{A}	6.64^{A}	60.35^{A}	13.42^{E}
FA with 0.50% solution	52.44^{B}	3.63^{B}	51.90^{B}	3.47^{B}	2.98^{B}	6.46^{B}	57.85^{B}	15.88^{D}
FA with 0.75% solution	49.30^{C}	2.96^{C}	49.33^{C}	3.38^{C}	2.93^{C}	6.32^{C}	54.57^{C}	18.97^{C}

FA with 1% solution	44.86 ^{D}	2.30 ^{D}	46.82 ^{D}	3.29 ^{D}	2.88 ^{D}	6.18 ^{D}	51.56 ^{D}	22.23 ^{B}
LSD (G) ($p \leq 0.05$)	1.20	0.25	1.95	0.06	0.04	0.07	2.27	1.12
LSD (S) ($p \leq 0.05$)	0.76	0.16	1.23	0.04	0.02	0.05	1.44	0.71
LSD (L) ($p \leq 0.05$)	1.21	0.24	1.95	0.07	0.04	0.07	2.28	1.12
G×S ($p \leq 0.05$)	NS	NS	NS	NS	0.05	NS	NS	NS
G×L ($p \leq 0.05$)	2.70	0.55	NS	NS	NS	NS	NS	NS
S×L ($p \leq 0.05$)	NS	NS	NS	NS	NS	NS	NS	NS
G×S× L ($p \leq 0.05$)	NS	NS	NS	NS	NS	NS	NS	NS

PH = Plant height; T = Tillers; EL = Electrolyte leakage; RWC = Relative water contents; S Zn = Shoot Zn contents; *CHL* a = Chlorophyll *a*; *CHL* b = Chlorophyll *b*; T *CHL* = Total Chlorophyll; any two means within a column followed by same letters are not significant at $p \leq 0.05$. n = 3; NS = non-significant; SP; Seed Priming; S_1 ($ZnSO_4.7H_2O$); S_2 ($ZnCl_2$)

Table 2. Correlation matrix of agronomic, water related and biochemical attributes of rice genotypes as influenced by the foliar application with different sources of zinc in pot experiment

Variable	EL	PH	RWC	S Zn	T	*CHL* a	*CHL* b
PH	-0.759**						
RWC	-0.780**	0.799**					
S Zn	-0.258**	0.172*	0.097^{NS}				
T	-0.805**	0.815**	0.682**	0.257**			
***CHL* a**	-0.086**	0.246**	0.145^{NS}	-0.088 ^{NS}	0.151 ^{NS}		
***CHL* b**	-0.346^{NS}	0.051^{NS}	0.015 ^{NS}	0.500**	-0.042 ^{NS}	-0.749**	
T *CHL*	-0.162*	0.241**	0.218**	0.629**	0.131 ^{NS}	0.173*	0.522**

PH = Plant height; EL = Electrolyte leakage; RWC = Relative water contents; S Zn = Shoot zinc contents; T = Tillers per plant; *CHL* a = Chlorophyll *a*; *CHL* b = Chlorophyll *b*; T *CHL* = Total chlorophyll; * = Significant at $p \leq 0.05$; ** = Significant at $p \leq 0.01$; NS = Non-significant

Table 3. Agronomic, water and biochemical attributes of rice genotypes as influenced by the soil application with different sources and levels of zinc in pot experiment

Factor	Agronomic Trait		Water related Trait	Biochemical Attribute				
	PH (cm)	T (per plant)	RWC (%)	*CHL* a (mg L^{-1})	*CHL* b (mg L^{-1})	T *CHL* (mg L^{-1})	S Zn (ppm)	EL (%)
Genotype (G)								
G_1 (Super Basmati)	54.34 ^{A}	2.66	55.12 ^{A}	3.29 ^{B}	1.72 ^{C}	5.02 ^{D}	42.18 ^{D}	15.03 ^{D}
G_2 (Accession-126)	43.01 ^{D}	2.30	44.30 ^{C}	3.05 ^{C}	4.11 ^{B}	7.17 ^{C}	60.95 ^{A}	19.55 ^{B}
G_3 (Accession-154)	42.11 ^{D}	2.40	43.82 ^{C}	3.25 ^{B}	0.65 ^{E}	3.90 ^{E}	50.80 ^{C}	21.31 ^{A}
G_4 (Accession-164)	47.35 ^{C}	2.56	50.62 ^{B}	0.50 ^{D}	7.24 ^{A}	7.74 ^{B}	60.40 ^{A}	18.21 ^{C}
G_5 (Accession-175)	52.60 ^{B}	2.73	52.48 ^{B}	7.09 ^{A}	1.03 ^{D}	8.12 ^{A}	56.52 ^{B}	17.75 ^{C}
Source (S)								
S_1	49.43 ^{A}	2.74 ^{A}	49.79	3.46 ^{A}	2.97 ^{A}	6.44 ^{A}	54.72	17.99 ^{B}
S_2	46.33 ^{B}	2.32 ^{B}	48.75	3.41 ^{B}	2.93 ^{B}	6.34 ^{B}	53.62	18.75 ^{A}
Level (L)								
Control	42.25 ^{E}	1.50 ^{D}	43.63 ^{E}	3.28 ^{E}	2.86 ^{E}	6.14 ^{E}	47.99 ^{E}	23.24 ^{A}
SA with 5 kg ha^{-1} of Zn	45.91 ^{D}	2.03 ^{C}	46.09 ^{D}	3.37 ^{D}	2.91 ^{D}	6.29 ^{D}	50.79 ^{D}	20.81 ^{B}
SA with 10 kg ha^{-1} of Zn	50.05 ^{B}	2.86 ^{B}	52.17 ^{B}	3.51 ^{B}	2.99 ^{B}	6.51 ^{B}	57.56 ^{B}	15.65 ^{D}
SA with 15 kg ha^{-1} of Zn	52.83 ^{A}	3.93 ^{A}	55.61 ^{A}	3.58 ^{A}	3.04 ^{A}	6.62 ^{A}	60.39 ^{A}	13.77 ^{E}
SA with 20 kg ha^{-1} of Zn	48.36 ^{C}	2.33 ^{C}	48.85 ^{C}	3.44 ^{C}	2.95 ^{C}	6.39 ^{C}	54.12 ^{C}	18.38 ^{C}
LSD (G) ($p \leq 0.05$)	1.01	NS	2.12	0.04	0.03	0.05	2.66	0.99
LSD (S) ($p \leq 0.05$)	0.64	0.24	NS	0.03	0.02	0.03	NS	0.63
LSD (L) ($p \leq 0.05$)	1.01	0.38	2.13	0.05	0.03	0.05	2.66	0.99
G×S ($p \leq 0.05$)	NS	NS	NS	NS	NS	NS	NS	NS
G×L ($p \leq 0.05$)	2.62	NS	NS	NS	NS	NS	NS	NS
S×L ($p \leq 0.05$)	NS	NS	NS	NS	NS	NS	NS	NS
G×S× L ($p \leq 0.05$)	NS	NS	NS	NS	NS	NS	NS	NS

PH = Plant height; T = Tillers; EL = Electrolyte leakage; RWC = Relative water contents; S Zn = Shoot Zn contents; *CHL* a = Chlorophyll *a*; *CHL* b = Chlorophyll *b*; T *CHL* = Total Chlorophyll contents; any two means within a column followed by same letters are not significant at $p \leq 0.05$. n = 3. NS = non-significant; SA: Soil Application; S_1 ($ZnSO_4.7H_2O$); S_2 ($ZnCl_2$)

Table 4. Correlation matrix of agronomic, water related and biochemical attributes of rice genotypes as influenced by the soil application with different sources and levels of zinc in pot experiment

Variable	*CHL* a	*CHL* b	EL	PH	RWC	S Zn	T *CHL*
***CHL* b**	-0.747**						
EL	-0.092^{NS}	-0.031^{NS}					
PH	0.323**	-0.123^{NS}	-0.704**				
RWC	0.163*	0.011^{NS}	-0.796**	0.780**			
S Zn	0.074^{NS}	0.471**	-0.224**	0.002^{NS}	0.243**		
T *CHL*	0.162*	0.534**	-0.164*	0.227**	0.225**	0.606**	
T	0.099^{NS}	-0.004^{NS}	-0.552**	0.574**	0.509**	0.290**	0.119^{NS}

PH = Plant height; EL = Electrolyte leakage; RWC = Relative water contents; S Zn = Shoot zinc contents; T = Tillers; *CHL* a = Chlorophyll *a*; *CHL* b = Chlorophyll *b*; T *CHL* = Total chlorophyll; * = Significant at $p \leq 0.05$; ** = Significant at $p \leq 0.01$; NS = Non-significant

Table 5. Growth attributes of rice genotypes as influenced by the different zinc application methods in field experiment

Factor	Growth attribute			
	LAI	CGR (g m^{-2} day^{-1})	DMP at flowering (kg ha^{-1})	DMP at maturity (kg ha^{-1})
Genotype (G)				
G$_1$ (Super Basmati)	4.90 ^{A}	64.12 ^{A}	9670 ^{A}	12780 ^{A}
G$_2$ (Accession-126)	4.43 ^{D}	53.78 ^{D}	9120 ^{D}	11540 ^{D}
G$_3$ (Accession-154)	4.07 ^{E}	48.23 ^{E}	8960 ^{E}	10780 ^{E}
G$_4$ (Accession-164)	4.61 ^{C}	58.32 ^{C}	9350 ^{C}	11890 ^{C}
G$_5$ (Accession-175)	4.78 ^{B}	61.34 ^{B}	9510 ^{B}	12100 ^{B}
Application Method (M)				
Control	4.06 ^{D}	50.54 ^{D}	9110 ^{D}	11100 ^{D}
Soil application (SA)	4.22 ^{C}	54.78 ^{C}	9270 ^{C}	11230 ^{C}
Foliar application (FA)	4.39 ^{B}	57.54 ^{B}	9390 ^{B}	11460 ^{B}
SA × FA	4.59 ^{A}	61.45 ^{A}	9650 ^{A}	11670 ^{A}
LSD (G) ($p \leq 0.05$)	0.13	1.76	0.09	0.10
LSD (M) ($p \leq 0.05$)	0.08	1.09	0.12	0.12
G×M ($p \leq 0.05$)	NS	NS	NS	NS

LAI = Leaf area index; CGR = Crop growth rate; DMP = Dry matter production

Any two means within a column followed by same letters are not significant at $p \leq 0.05$. n = 3. NS = non-significant

Table 6. Biochemical and yield attributes of rice genotypes as influenced by the different zinc application methods in field experiment

Factor	Biochemical and yield attribute				
	CHL a (mg L^{-1})	*CHL* b (mg L^{-1})	EL (%)	Grain yield (kg ha^{-1})	Grain Zn contents (ppm)
Genotype (G)					
G$_1$ (Super Basmati)	3.42 ^{B}	1.80 ^{C}	14.96 ^{D}	4530 ^{A}	27.86 ^{E}
G$_2$ (Accession-126)	3.11 ^{C}	4.17 ^{B}	19.90 ^{B}	3260 ^{D}	59.12 ^{A}
G$_3$ (Accession-154)	3.37 ^{B}	0.69 ^{E}	21.23 ^{A}	3060 ^{E}	45.19 ^{D}
G$_4$ (Accession-164)	0.64 ^{D}	7.31 ^{A}	17.86 ^{C}	3570 ^{C}	55.26 ^{B}
G$_5$ (Accession-175)	7.23 ^{A}	1.05 ^{D}	17.89 ^{C}	4060 ^{B}	50.59 ^{C}
Application Method (M)					
Control	3.46 ^{C}	2.92 ^{D}	23.17 ^{A}	3190 ^{D}	43.12 ^{D}
Soil application (SA)	3.53 ^{B}	2.98 ^{C}	20.23 ^{B}	3460 ^{C}	46.29 ^{C}
Foliar application (FA)	3.59 ^{AB}	3.03 ^{B}	17.05 ^{C}	3690 ^{B}	48.16 ^{B}
SA × FA	3.64 ^{A}	3.09 ^{A}	13.06 ^{D}	4280 ^{A}	52.61 ^{A}
LSD (G) ($p \leq 0.05$)	0.07	0.02	0.91	0.12	0.95
LSD (M) ($p \leq 0.05$)	0.06	0.01	0.80	0.11	0.86
G×M ($p \leq 0.05$)	NS	NS	NS	NS	NS

CHL a = Chlorophyll *a*; *CHL* b = Chlorophyll *b*; EL = Electrolyte leakage;

Any two means within a column followed by same letters are not significant at $p \leq 0.05$. n = 3. NS = non-significant

Table 7. Body weight of groups of male rats after feeding with Zn biofortified rice in animal feeding experiment

Treatment	I.B.W. (g)	1st week (g)	2nd week (g)	3rd week (g)	4th week (g)
27 mg/kg of Zn	95.45	109.82 ^{D}	125.83 ^{D}	137.04 E	151.57 ^{D}
45 mg/kg of Zn	96.03	118.73 ^{C}	138.40 ^{C}	152.13 ^{C}	164.64 ^{C}
50 mg/kg of Zn	95.38	122.84 ^{B}	142.70 ^{B}	156.07 ^{B}	169.89 ^{B}
55 mg/kg of Zn	96.23	129.13 ^{A}	145.54 ^{A}	162.88 ^{A}	174.79 ^{A}
59 mg/kg of Zn	95.94	117.50 ^{C}	135.53 ^{C}	148.81 ^{D}	163.50 ^{C}
LSD Value		**2.44**	**2.56**	**2.40**	**4.13**

Data is average of 8 repeats and different letters in column show statistically significant differences at $p \leq 0.05$
I.B.W. = Initial body weight

Table 8. Hematological parameters of groups of male rats after feeding with Zn biofortified rice in animal feeding experiment

Treatment	RBC (10^{12}/l)	WBC (10^{9}/l)	HGB (g/dl)	HCT (%)	MCV (fl)	MCH (pg)	MCHC (g/dl)
27 mg/kg of Zn	5.16	12.54 C	12.37	31.16	62.37	26.88	34.76
45 mg/kg of Zn	5.17	14.17 B	12.76	31.50	62.53	26.24	35.67
50 mg/kg of Zn	5.18	14.77 B	13.04	31.58	63.14	26.27	34.48
55 mg/kg of Zn	5.18	15.81 A	12.59	31.61	63.07	26.18	34.81
59 mg/kg of Zn	5.18	11.17 D	12.87	31.96	62.60	25.87	35.11
LSD Value	**NS**	**0.96**	**NS**	**NS**	**NS**	**NS**	**NS**

RBC = Red blood cell; WBC = White blood cells; HGB = Hemoglobin; HCT = Hematocrit; MCV = Mean corpuscular volume; MCH = Mean corpuscular hemoglobin; MCHC = Mean corpuscular hemoglobin concentration
Data is average of 8 repeats and different letters in column show statistically significant differences at $p \leq 0.05$

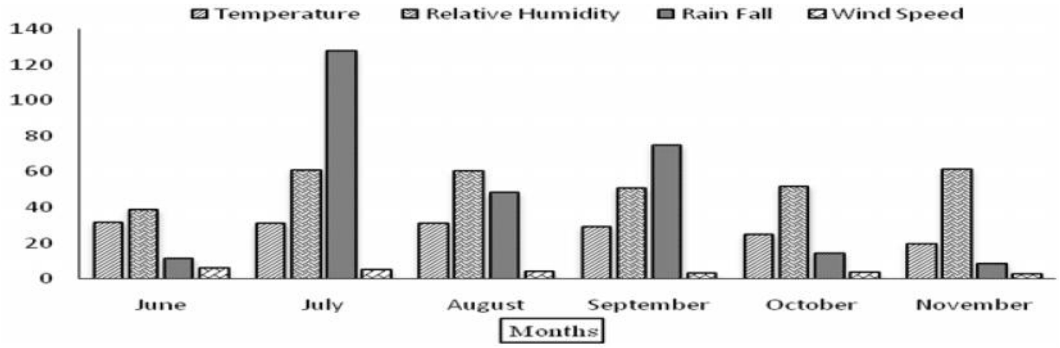
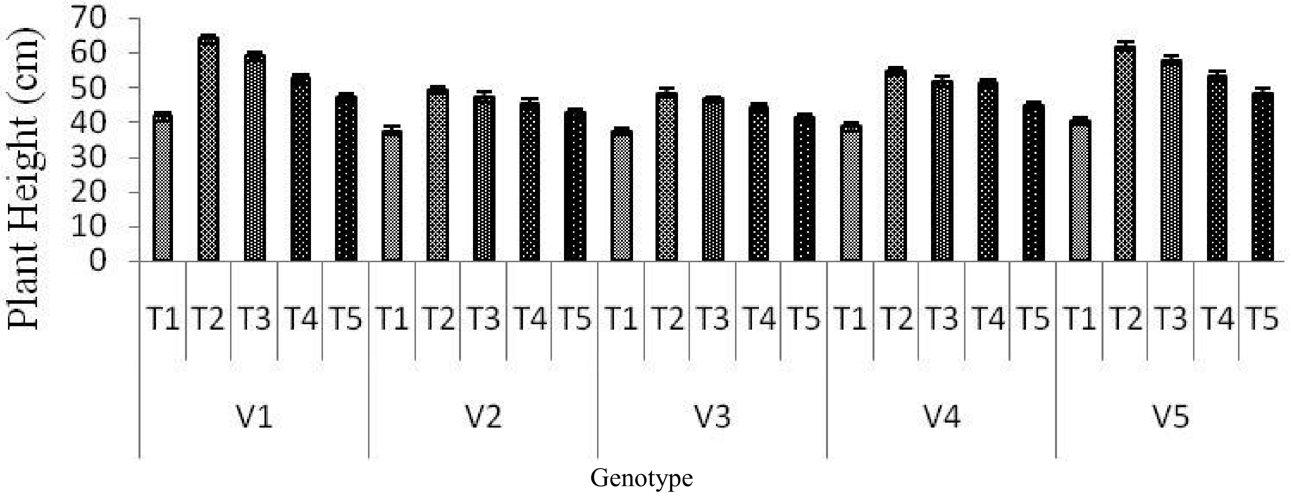
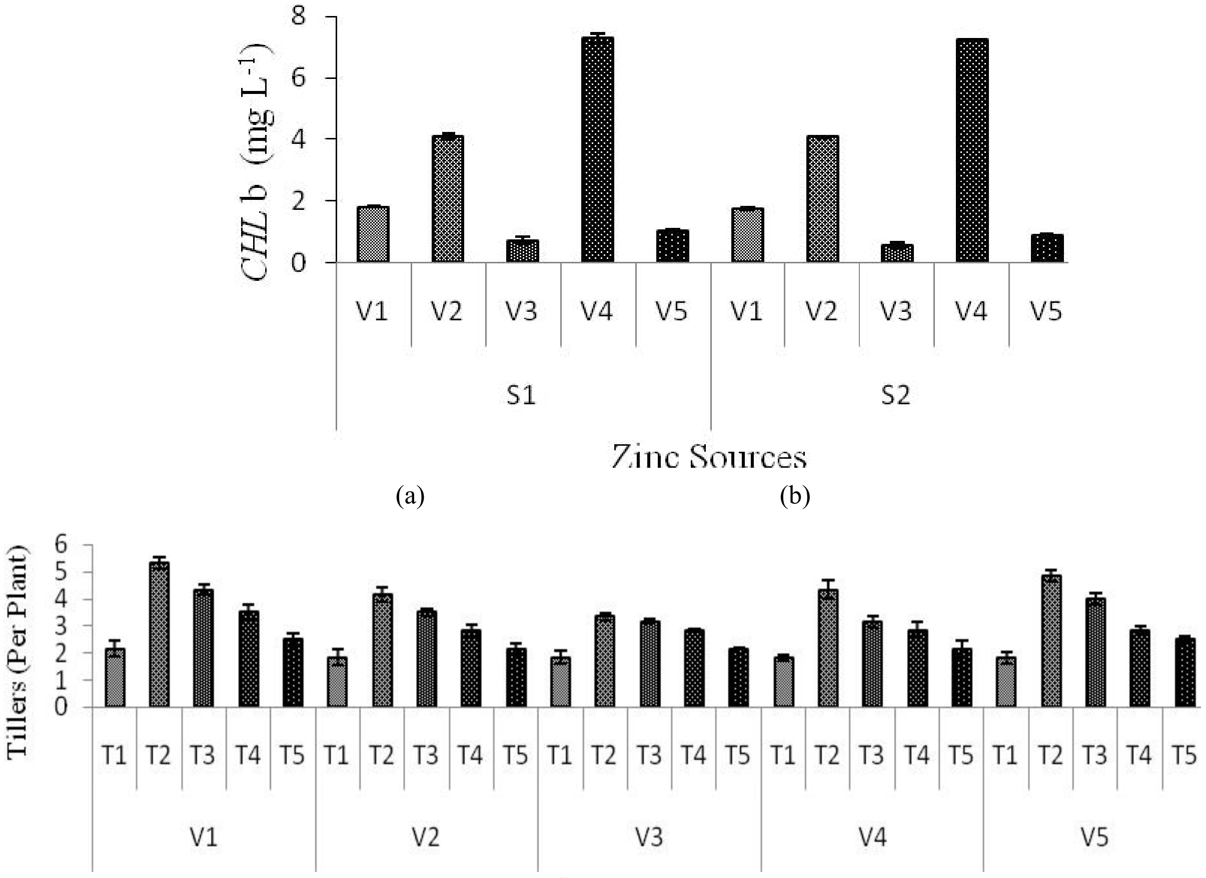


Fig. 1. Prevailing climatic conditions of the experimental site during crop growing season.





(a) (b)

(c)

Fig 2. Interactive effect of a) Genotypes × Levels of Zinc; b) Genotypes × Zinc sources; c) Genotypes × Levels of Zinc on the plant height (cm), *CHL* b (mg L^{-1}) and total tillers (per plant) respectively, as influenced by the foliar application with different sources and levels of zinc in pot experiment

FA: Foliar Application; S_1 ($ZnSO_4.7H_2O$); S_2 ($ZnCl_2$); T_1 (Control); T_2 (FA of 0.25% solution); T_3 (FA of 0.50% solution); T_4 (FA of 0.75% solution); T_5 (FA of 1% solution)

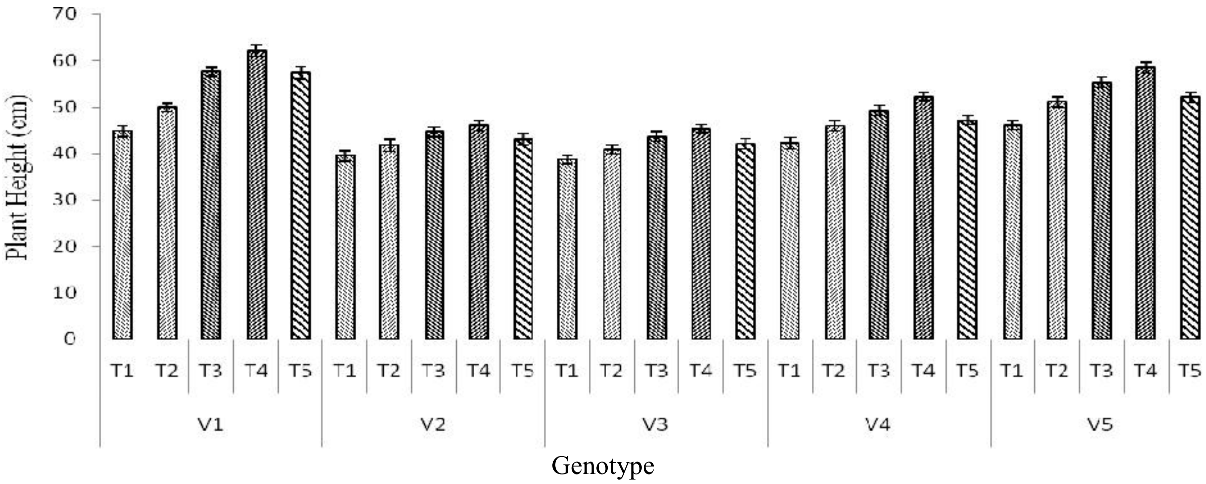


Fig 3. Interactive effect of Genotypes × Levels of Zn on the plant height (cm) as influenced by the soil application with different sources and levels of zinc

SA: Soil Application; S_1 ($ZnSO_4.7H_2O$); S_2 ($ZnCl_2$); T_1 (Control); T_2 (SA of 5 kg ha^{-1}); T_3 (SA of 10 kg ha^{-1}); T_4 (SA of 15 kg ha^{-1}); T_5 (SA of 20 kg ha^{-1}).

DISCUSSION

The results of pot studies revealed that Zn application in rice genotypes by various methods, using different sources particularly at low concentrations, improved crop stand establishment, tillering ability, photosynthetic pigments and water relations. Foliar as well as soil application with $ZnSO_4.7H_2O$ also improved early seedling growth in rice genotypes by improving the agronomic traits in relation to water and biochemical attributes. The increase in agronomic traits (plant height and tillers per plant) by the $ZnSO_4$ application at 15 kg ha^{-1} soil applied and 0.25% in the form of foliar application was found best. This increase in the plant height and tillering dynamics was probably because Zn^{2+} plays an important role in plants metabolism. It is involved in auxin metabolism, synthesis of cytochrome C and stabilization of ribosomal fraction (Zou *et al.,* 2012). Increase in number of productive tillers might be due to adequate supply of Zn and its availability (Begum *et al.,* 2016). The difference among genotypes may be due to difference in their genetic makeup. Maret, (2013) has already reported that Zn is necessary for cell division and elongation processes. Stunted growth and small leaves are the most distinct Zn deficiency symptoms which are possibly due to changes in auxin metabolism, particularly of IAA (Begum *et al.,* 2016). Similar, results were reported by Wang *et al.* (2015); they observed that adequate supply of Zn increased the plant height and total tillers of fine rice.

Zinc also provides building material for leaf chlorophyll contents. Foliar application with 0.25% solution and soil application at 15 kg ha^{-1} using $ZnSO_4$ improved the chlorophyll *a*, *b* and total chlorophyll contents of all rice genotypes showing involvement of Zn in chlorophyll synthesis (Abadi and Sepehri, 2016). Reduction in the chlorophyll contents at low zinc might be due to low zinc/or magnesium because zinc does not have any direct role in the chlorophyll formation but it effects the concentration of those nutrients involved in chlorophyll formation or which are the part of chlorophyll molecule (Fe and Mg). The increasing trend in chlorophyll a, b and total chlorophyll content of rice genotypes due to the increase of rice quality by the Zn^{2+}. The present study has revealed the similar result as reported earlier by (Ramesh *et al.,* 2014).

Gradual decrease in RWC with increasing Zn concentration was observed whereas highest level was noticed where foliar application at 0.25% and soil application at 15 kg ha^{-1} using $ZnSO_4$. These results indicated dependency of RWC contents on Zn. It is obvious that RWC increased at steady level then declined thereafter. These results help to know about optimum and toxicity level of Zn that is to be recognized for rice genotypes. Improvement in water relations due to Zn application might be due to increased water uptake through increased number of root tips which may have been resulted in more water uptake by the plants helping them to grow vigorously. Due to better development of root system plants enable to extract water efficiently resulting better water relations as observed in this study. Previous reports also affirm that Zn deficiency in leaves led to have poor water status than leaves with normal Zn concentration (Zhao *et al.,* 2016). Generation of free radicals and increased lipid per oxidation under zinc stress (deficiency or toxicity) may have resulted in loss of membrane integrity and increase in membrane permeability (Samreen *et al.,* 2013). Jain *et al.* (2010) reported that elevated levels of H_2O_2 and O_2 caused by stress assist the formation of active radicals like (OH).

In this study, zinc application significantly improved LAI, CGR and DMP of all the rice genotypes. This increase in the growth attributes might be due to the promoting plant growth and development by better uptake of nutrients. This increase in LAI with Zn application is a settlement with the work of previous study of Zhou *et al.* (2016) who reported that zinc sulphate application positively impacted growth of plants as a result of increase in chlorophyll, increase in photosynthetic rate resulting better leaf area index. Similar finding has been given by Tariq *et al.* (2014) who reported that applied Zn increased the photosynthesis and chlorophyll production, ultimately increasing the dry weight CGR and total yield. However, without Zn application there is less improvement in LAI, CGR, and DMP in all rice genotypes. The results showed positive relation with the earlier study that decline in sunlight intervention because of lower leaf area and lessening in carbon fixation/unit of leaf area or to destruction of the photosynthetic machine (Marschner, 2012).

The results showed that application of Zn increased the kernel yield over control, which might be attributed to the better enzymatic activity and auxin metabolism in plants by Zn (Zhou *et al.,* 2016). In this study, improvement in the kernel yield might be due to better tillering capacity, improved panicle length and 1000-kernle weight in all the rice genotypes. Khan *et al.* (2012) reported maximum panicle length, kernel per panicle and grain yield when Zn was applied at 9 kg ha^{-1}. The increase in kernel yield might be due to involvement of Zn in numerous biochemical processes in rice plants such as nucleotides production, auxin metabolism, enzyme activation, chlorophyll formation protein synthesis (Abaid-Ullah *et al.,* 2015), carbohydrates, lipids and nucleic acids metabolism, gene expression (Gomez-Coronado *et al.,* 2016; Yavas and Unay, 2016) and regulation and pollen formation and a special role in fertilization as pollen grains have a very high concentration of Zn.

In the pot experiments, shoot Zn concentration ranged from 47.0 to 60.5 ppm in various Zn application treatments. A concentration greater than 50 ppm Zn in rice plant is generally considered desirable for achieving a positive impact in the grain Zn concentration that will ultimately cause a positive impact on human health for combating malnutrition (Zaman *et al.,* 2017).

Foliar application beyond 0.25% and soil application beyond 15 kg ha^{-1} was toxic as seedling growth was suppressed (Tables 1, 3). Moreover, the deficiency and higher levels of Zn cause discoloration of the foliage, seedling growth restriction as Zn toxicity suppresses cell division and necrosis of upper leaves (Monreal *et al.,* 2015). At optimum concentration, Zn has been found to activate key enzymes involved in starch metabolism e.g. starch phosphorylase, α-amylase etc (Maret, 2013). Nonetheless, application of high or too low Zn to the plants proved to be toxic that ultimately results in poor synthesis of plant proteins (Begum *et al.,* 2016). Moreover, higher concentration of Zn may diminish root and leaf development owing to substantial decrease in NADPH (Nicotinamide adenine dinucleotide phosphate) production in chloroplasts (Mousavi, 2011). In the field study, Zn concentration in shoot ranged from 42 to 62 ppm under different Zn applications. Phattarakul *et al.* (2012) reported that Zn concentration in brown rice was increased by 25% and 32% by foliar and foliar + soil Zn applications as compared with control, respectively. Foliar + soil Zn application significantly increased Zn concentration in rice kernels. Foliar Zn applied is easily absorbed and transported through phloem (Jan *et al.,* 2016). Increase in Zn concentration of all rice genotypes would provide significant nutritional benefits to rice consumers, particularly for those who have limited access to Zn from other food sources. High grain Zn has also important agronomic benefits for plants grown under low Zn supply. The combined application of foliar + soil is superior for both grain yield and grain Zn intake (Gomez-Coronado *et al.,* 2016). There are nearly 2800 proteins which need Zn for their structural and functional integrity (Feinauer *et al.,* 2013). These findings indicate that there may be high need for Zn during root and coleoptiles development, for active protein synthesis and/or other related functions. During soil and foliar application in the pot study, use of $ZnSO_4$ as a Zn source was a better option than $ZnCl_2$ (Tables 1, 3). Poor seedling growth was observed where foliar application with $ZnCl_2$ was done. This indicates the possible toxic effects of Cl^- on photosynthesis and cellular respiration (Stanković *et al.,* 2011; Hippler *et al.,* 2015).

In the rat feeding experiment, lesser body weight gain was observed in those albino rats fed on zinc deficient diet compared to those fed diet containing sufficient amount of zinc. These results are in accordance with Della Lucia *et al.* (2014) who stated that enhanced Zn dietary intake would result in an increase in the weight gain of test animals. These findings imply that deficiency of Zn has role in metabolism of mammals and reduction in the hunger and ability of rats to consume food intake as in those rats which were fed to Zn sufficient diet. In another trial the food conversion efficiency of Zn-sufficient animals was higher than Zn-deficient animals (Lazarte *et al.,* 2015). The body weight gain (BWG) of the rats might be associated with feed intake going by the corresponding trend in weight increase with intake per day. The poor performance in weight gain of rats fed diets and the death of rats fed diets might be likely due to low quantity of feed they consumed. However, the increase in the WBC clearly indicates that those rats are less susceptible to diseases and better intake of food increased the body weight of those albino rats. In conclusion, soil + foliar application at 15 kg ha^{-1} and 0.25% solution using $ZnSO_4$ improved the crop stand establishment, water relations, maintaining the membrane permeability, photosynthetic pigments, growth attributes, kernel yield and its kernel biofortification.

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