EFFICACY OF ZINC APPLICATION METHODS FOR CONCENTRATION AND ESTIMATED BIOAVAILABILITY OF ZINC IN GRAINS OF RICE GROWN ON A CALCAREOUS SOIL

Muhammad Imran[1,2,3], Shamsa Kanwal[1], Shahid Hussain[1,2,*], Tariq Aziz[1,4] and Muhammad Aamer Maqsood[1]

[1]Institute of Soil and Environmental Sciences, University of Agriculture, Faisalabad-38040, Pakistan
[2]Department of Soil Science, Bahauddin Zakariya University, Multan-60800, Pakistan
[3]Department of Soil Science, Muhammad Nawaz Shareef University of Agriculture, Multan-61000, Pakistan
[4]School of Plant Biology, University of Western Australia, Crawley, WA 6009, Australia
*Corresponding authors e-mail: shahid.hussain@bzu.edu.pk

Zinc biofortification of cereal grains is suggested for controlling widespread human Zn deficiency in developing countries. In present field trail, various Zn treatments [control, priming of seeds in 0.5% (w/v) Zn solution (seed priming), dipping of roots in 0.5% (w/v) Zn solution (root dipping), application of 20 kg Zn ha^{-1} (soil application), sprays of 0.25% (w/v) Zn each at tillering and heading stages (foliar application) and combination of soil + foliar Zn applications] were applied as $ZnSO_4 \cdot 7H_2O$ to rice grown under submerged conditions on a Zn deficient calcareous soil. Treatments significantly ($P \leq 0.05$) increased grain and straw yield; however, seed priming and root dipping increased paddy yield only by $\leq 5\%$. Increased grain weight also significantly increased grain phytate content. Whole grain Zn concentration increased from 22 (at control) to 29 mg kg^{-1} (at soil + foliar application).Zinc applications methods, especially soil + foliar application, decreased grain [phytate]:[Zn] ratio and increased estimated human Zn bioavailability in grains based on trivariate model of Zn absorption. Conclusively, soil + foliar Zn application is suitable for optimum paddy yield and agronomic Zn biofortification of rice grains. However, a limited increase in grain Zn concentration (7 mg kg^{-1}) by Zn application suggested exploitation of molecular and genetic approaches in Zn biofortification programs.
Keywords: biofortification, calcareous soil, human bioavailability, rice, zinc

INTRODUCTION

Zinc is essential for all biological systems i.e., humans, animals and plants. In plants, Zn serves in a variety of biochemical processes such as nucleotide, cytochrome synthesis, chlorophyll production, auxin metabolism, membrane integrity, and enzyme activation (Broadley *et al.*, 2007). Similarly in humans, it plays an essential role in normal growth and reproduction. However, almost half of the world's human population is thought to be affected from Zn insufficiency as a result of low Zn intake (WHO, 2002; Assunção *et al.*, 2010). In Pakistan, one third of children and 40% of mothers are Zn deficient and occurrence of Zn insufficiency in Pakistan is more frequent in rural community (MINH, 2009).

Zinc insufficiency in soils is also recognized as widespread nutritional problem throughout the rice growing countries. This is often related to low phyto-availability and high fixation of Zn due to high pH, calcareousness and submerged soil conditions (Alloway, 2009; Hussain *et al.*, 2011; Rehman *et al.*, 2012). In Pakistan, soil Zn deficiency in rice growing areas of the country is well established and about 70% of rice belt is estimated to be Zn deficient (Hamid and Ahmad, 2001).

Rice (*Oryza sativa* L.) is an important staple food and provides about 20% of total calories consumed by the world's population (FAOSTAT, 2014). Like other cereals, it is a poor source of various minerals like Zn. In addition to reduction in yield, low supply of Zn from soils also related with low grain Zn concentration (Alloway, 2009). To produce economical yields and rice grains high in Zn concentration, Zn application is recommend for soils low in Zn availability (Rehman *et al.*, 2012).

Zinc can be applied by different methods; such as soil application, foliar application and root dipping (Johnson *et al.*, 2005; Cakmak, 2008). Soil application is most successful for continuous supply of nutrients which are required in greater amounts. On the other hand, foliar Zn sprays are more effective for achieving a quick remedy to plant Zn deficiency in conditions of low Zn supply from soils. Seed priming is also reported to increase paddy yield and grain Zn concentration in cereals (Harris *et al.*, 2008; Farooq *et al.*, 2006). Previously, many researchers have compared various Zn application methods for optimum paddy yield of rice (Rehman *et al.*, 2012). However, comparative efficiency of different Zn application methods for Zn biofortification in rice grains is rarely reported for alkaline calcareous soils. In flooded conditions, Zn

deficiency is aggravated because of changes in chemistry of soils (Alloway, 2009); hence appropriate dose, method and time of application are very important topics in Zn nutrition of rice.

In present study, rice crop was applied with different Zn treatments (seed priming, root dipping, soil application, foliar application and soil + foliar application) and it was hypothesized that treatments will differentially affect paddy yield and grain Zn concentration.

A phosphorous storing compound, phytate, in cereals grains complexes with metal cations, including Zn, and hinders their absorption for human consumption (Weaver and Kannan, 2002). To better understand the effect of Zn treatments on grain quality and its suitability for human Zn supply, grain phytate concentration was also measured along with computations for human Zn bioavailability based on [phytate]:[Zn] ratios and trivariate model (Brown *et al.*, 2001; Miller *et al.*, 2007).

MATERIALS AND METHODS

Growth conditions: A field trail was carried out at Research Farm (31.439° N, 73.069° E) of Institute of Soil and Environmental Sciences, University of Agriculture, Faisalabad (Pakistan). Before sowing, randomized soil samples (0–15 cm depth) were collected for soil characterization. Collected soil was air-dried, passed into 2 mm sieve and mixed thoroughly. Representative subsamples of the soil were analyzed in laboratory for physicochemical characteristics (Table 1).

Rice cv. *Basmati-515* was tested for following Zn treatments under field condition: control; seed priming (before sowing of nursery, one kg of seeds were soaked overnight in one L of 0.5% w/v Zn solution); root dipping (at the time of transplantation, roots of 1500 seedlings were dipped in 7.5 L of 0.5% w/v Zn solution for 2 h); soil application (20 kg Zn ha^{-1} was applied to plots at the time of nursery transplantation); foliar application (0.25% w/v Zn solution at 400 L ha^{-1} was sprayed each at 25 days after transplantation and heading stage); and combination of soil + foliar

treatments. Twenty-five day old rice seedlings were transplanted to experimental plots and Zn treatments were applied to the soil and respective parts of the plants. Source of Zn for all treatments was hydrated zinc sulphate ($ZnSO_4·7H_2O$) while foliar sprays also contained 0.01 % (v/v) Tween as surfactant. The six Zn treatments were arranged in randomized complete block design. The experimental area was divided in to four blocks with total of 24 plots each of 3 m × 5 m size and 296 healthy rice seedling of uniform growth were transplanted per plot by maintaining row-to-row and plant-to-plant distance of 22.5 cm.

At transplantation, plots were uniformly supplied (in kg ha^{-1}) with 100 N, 67 P and` 60 K by applying urea, di-ammonium phosphate and potassium sulphate. A second dose of 60 kg N ha^{-1} was applied 25 days after transplantation. During crop growth, canal water was used to maintain submerged soil conditions.

Crop harvest and chemical analysis: Whole plots were harvested at maturity and manually threshed to separately measure straw and paddy yields. Subsamples of grains from each plot were washed with distilled water and rapidly dried with tissue papers before oven drying at 65$^{°}$C for 72 hours. Then, these samples were finely ground in a mill (*IKA WERKE*, *MF 10 Basic*, Staufen, Germany). Ground subsamples of known weights were wet digested in a di-acid mixture (HNO_3:$HClO_4$ ratio of 2:1) (Jones and Case 1990). Zinc concentration was measured in the digest by an atomic absorption spectrophotometer (PerkinElmer, Aanalyst 100, Waltham, USA). Phytate from rice grains was extracted with 10 mL of 0.2 N HCl at room temperature after shaking the mixture continuously for 2 h. Phytate in the extract was determined by an indirect method (Haug and Lantzsch, 1983) on a spectrophotometer (Shimadzu, UV-1201, Kyoto, Japan) (Hussain *et al.*, 2012a).

Human Zn bioavailability in grains: Bioavailability of Zn from a diet depends on concentration of both Zn and phytate; therefore, Zn bioavailability to humans was qualitatively estimated as molar ratio of phytate to Zn ([phytate]:[Zn] ratio) in rice grains (Brown *et al.*, 2001; Weaver and Kannan

Table 1. Selected physical and chemical properties of soil in the field

Soil characteristic		Unit	Value	Method
Textural class		---	Loam	USDA classification
Particle size distribution	Sand	g kg^{-1}	429±8	Hydrometer method (Gee and Bauder, 1986)
	Silt		393±10	
	Clay		178±4	
pH$_s$		---	7.8±0.1	pH of saturated soil paste
EC$_e$		dS m^{-1}	1.75±0.05	Electric conductivity of saturated soil paste extract
Organic matter		g kg^{-1}	6.5±0.6	Walkley-Black method (Jackson, 1962)
Calcium carbonate		g kg^{-1}	4.6±0.2	Acid dissolution (Allison and Moodie, 1965)
Plant available Zn		mg kg^{-1}	0.37±0.03	Extracted with 0.005 *M*DTPA

Five samples were analyzed after random composite sampling

2002). To have a quantitative estimate of Zn bioavailability, trivariate model of Zn absorption was employed to calculate human Zn bioavailability (Miller *et al.*, 2007).

$$TAZ = 0.5 \cdot \left(A_{MAX} + TDZ + K_R \cdot \left(1 + \frac{TDP}{K_P} \right) - \sqrt{\left(A_{MAX} + TDZ + K_R \cdot \left(1 + \frac{TDP}{K_P} \right) \right)^2 - 4 \cdot A_{MAX} + TDZ} \right)$$

Where, A_{MAX} (maximum Zn absorption) = 0.091, K_R (equilibrium dissociation constant of zinc-receptor binding reaction) = 0.680 and K_P (equilibrium dissociation constant of Zn-phytate binding reaction) = 0.033 relate to Zn homeostasis in human intestine (Hambidge *et al.*, 2010). In the model, total daily absorbed Zn (TAZ) (mg Zn d^{-1}) is a function of total daily dietary phytate (TDP) (mmol phytate d^{-1}) and total daily dietary Zn (TDZ) (mmol Zn d^{-1}).

Statistical analysis: Analysis of variance (ANOVA) was based on completely randomized block design and treatment were ranked by Least Significant Difference (LSD) test at $P \leq 0.05$ (Steel *et al.*, 1997). Various statistical computations were run on *Statistix 9® for Windows* (Analytical Software, Tallahassee, USA).

RESULTS

Straw and paddy yields: Zinc application significantly ($P \leq 0.05$) increased straw yield as compared to control (Table 2). Maximum increase of 28% was observed in soil+foliar application of Zn followed by 22% increase with soil Zn application alone. While, minimum increase in straw yield was observed with seed priming and root dipping treatments.

Paddy yield of rice was also significantly ($P \leq 0.05$) influenced by various Zn treatments (Table 2). Similar to straw yield, the paddy yield was maximum (4.8 t ha^{-1}) with soil + foliar application of Zn followed by sole applications to soil and foliage, respectively. Whereas, seed priming did not significantly influence paddy yield of rice.

Only soil, foliar and soil + foliar Zn treatments significantly ($P \leq 0.05$) increased grain weight seed^{-1} (Table 2). Again, it was maximum (32% greater than control treatment) with soil + foliar Zn application followed by sole Zn application to soil and foliage, respectively.

Zinc and phytate in grains: Various Zn applications, except seed priming, significantly ($P \leq 0.05$) increased grain Zn concentration over control (Fig. 1a). Depending on the Zn treatment, grain Zn concentration ranged from 22 (at control) to 29 mg kg^{-1} (at soil + foliar Zn application). However, soil Zn application alone and root dipping alone had only a medium effect on grain Zn concentration.

Similar to grain Zn concentration, maximum Zn content seed^{-1} (59% greater than control treatment) was achieved with soil + foliar Zn application (Fig. 1b) followed by Zn applications to soil alone or foliage alone. Root dipping also significantly increased grain Zn content over 0 Zn applied control. Significance of Zn treatments for grain Zn content

(a)

Table 2. Straw and paddy yields of rice crop grown in field and differentially treated with Zn

Zn treatments	Straw yield (t ha^{-1})	Paddy yield (t ha^{-1})	Seed weight (mg seed^{-1})
Control	8.9 E	3.9 E	20.9 D
Seed priming	10.0 D	4.0 DE	20.7 D
Root dipping	9.8 D	4.1 D	21.6 D
Soil	10.9 B	4.6 B	25.5 B
Foliar	10.4 C	4.4 C	23.4 C
Soil + Foliar	11.4 A	4.8 A	27.6 A

Zinc treatments included: 0 Zn applied control (Control), priming of seeds in 0.5% w/v Zn solution (Seed priming), dipping of roots in 0.5% w/v Zn solution (Root dipping), soil application of 20 kg Zn ha^{-1} (Soil), two sprays of 0.5% (w/v) Zn (foliar application) and combination of soil + foliar Zn applications (Soil + Foliar). Different letters in the same column indicate significant differences by LSD at $P \leq 0.05$.

Table 3. Concentration and content of phytate in grains of rice crop grown in field and differentially treated with Zn

Zn application	Concentration (mg phytate g^{-1})	Content seed^{-1} (mg phytate seed^{-1})	Content ha^{-1} (kg phytate ha^{-1})
Control	14.8 A	0.31 B	58 D
Seed priming	14.8 A	0.31 B	61 CD
Root dipping	14.7 A	0.32 B	64 CD
Soil	13.4 B	0.34 A	73 AB
Foliar	13.4 BC	0.31 B	66 BC
Soil + Foliar	12.7 C	0.35 A	75 A

Zinc treatments included: 0 Zn applied control (Control), priming of seeds in 0.5% w/v Zn solution (Seed priming), dipping of roots in 0.5% w/v Zn solution (Root dipping), soil application of 20 kg Zn ha^{-1} (Soil), two sprays of 0.5% (w/v) Zn (foliar application) and combination of soil + foliar Zn applications (Soil + Foliar). Different letters in the same column indicate significant differences by LSD at $P<0.05$.
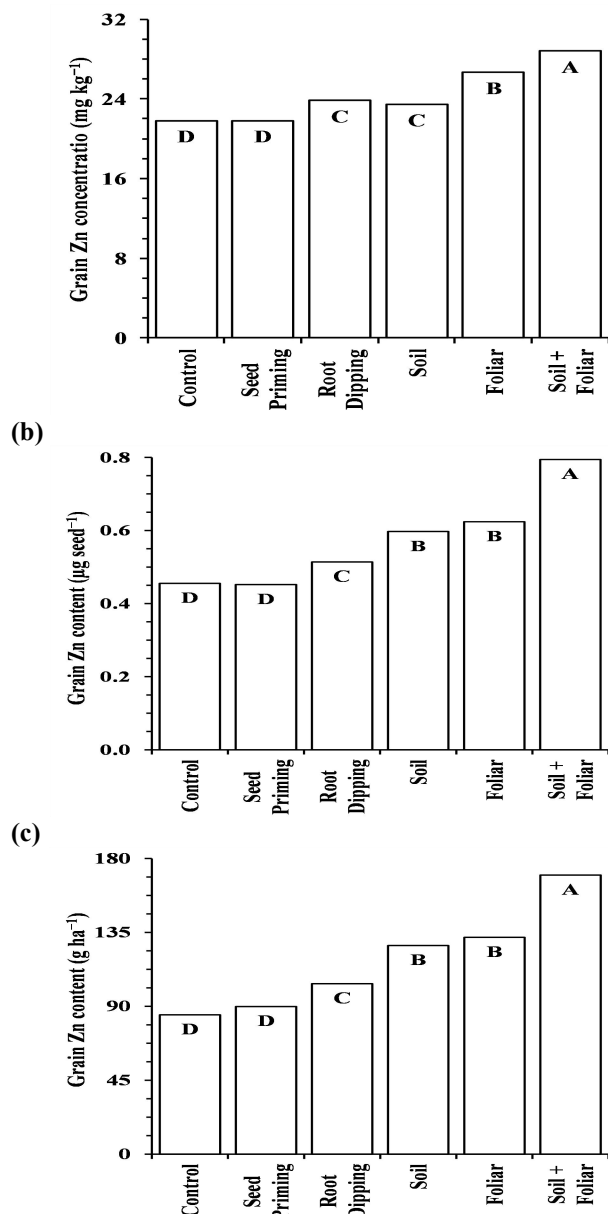
(b)

(c)

Figure 1. Grain Zinc concentration (a), content seed^{-1} (b) and content ha^{-1}(c) in grains of rice crop grown in field and differentially treated with Zn. Zinc treatments included: 0 Zn applied control (Control), priming of seeds in 0.5% w/v Zn solution (Seed priming), dipping of roots in 0.5% w/v Zn solution (Root dipping), soil application of 20 kg Zn ha^{-1} (Soil), two sprays of 0.5% (w/v) Zn (foliar application) and combination of soil + foliar Zn applications (Soil + Foliar). Different letters in the same pan indicate significant differences by LSD at P≤0.05.

ha^{-1} (Fig. 1c) was similar as described above for Zn content seed^{-1} (Fig. 1b).

Grain phytate concentration ranged from 12.7 to 14.8 mg g^{-1} under various Zn treatments (Table 3). Only soil Zn application, foliar Zn application and their combination significantly ($P≤0.05$) decreased phytate concentration in rice grains. Decrease in grain phytate concentration was 9% with soil Zn application, 10% with foliar Zn application and 14% with their combination.

Phytate content seed^{-1} was significantly ($P≤0.05$) influenced only when soil Zn was included in treatments (Table 3). Similarly, Zn treatments significantly influenced phytate content ha^{-1}. Compared with phytate content ha^{-1} at foliar Zn application, phytate content ha^{-1} was significantly greater at soil + foliar Zn application (14%) and significantly lower at control (12%). However, phytate content ha^{-1}achieved with soil application was statistically at par with phytate content ha^{-1}achieved with foliar and soil + foliar applications of Zn.

Estimated human Zn bioavailability in grains: Various Zn applications, except seed priming, significantly ($P≤0.05$) decreased grain [phytate]:[Zn] ratio over control (Fig. 2a). Minimum [phytate]:[Zn] ratio of 44 (35% less than control) in rice grains was achieved with soil + foliar Zn application followed by sole foliar Zn application.

Zinc treatments also significantly ($P≤0.05$) influenced trivariate model of Zn absorption based estimated Zn bioavailability in rice grains (Fig. 2b). Contrary to [phytate]:[Zn] ratio, estimated Zn bioavailability was minimum (1.1 mg Zn for 300 g rice grains) at 0 Zn applied control and maximum (1.5 mg Zn for 300 g rice grains) at soil + foliar Zn application. As compared to control, soil Zn application increased estimated Zn bioavailability by 12%; however, this increase was statistically similar to that achieved with root dipping. Foliar application increased estimated Zn bioavailability in rice grains by 25%.

DISCUSSION

Most of Pakistani soils are alkaline calcareous in nature and soil under the trail was also alkaline calcareous (Table 1). These soils are generally Zn deficient; having <1.0 mg of DTPA extractable Zn kg^{-1} soil (Alloway, 2008; Hamid and Ahmad, 2001). Therefore, application of Zn to the soil (which had 0.37 mg Zn kg^{-1} soil, see table 1) significantly ($P≤0.05$) increased straw and paddy yields of rice (Table 2). This has previously been reported by a number of researchers for rice and other crops (Harris *et al.*, 2008; Hussain *et al.*, 2012a and b; Khan *et al.*, 2009).
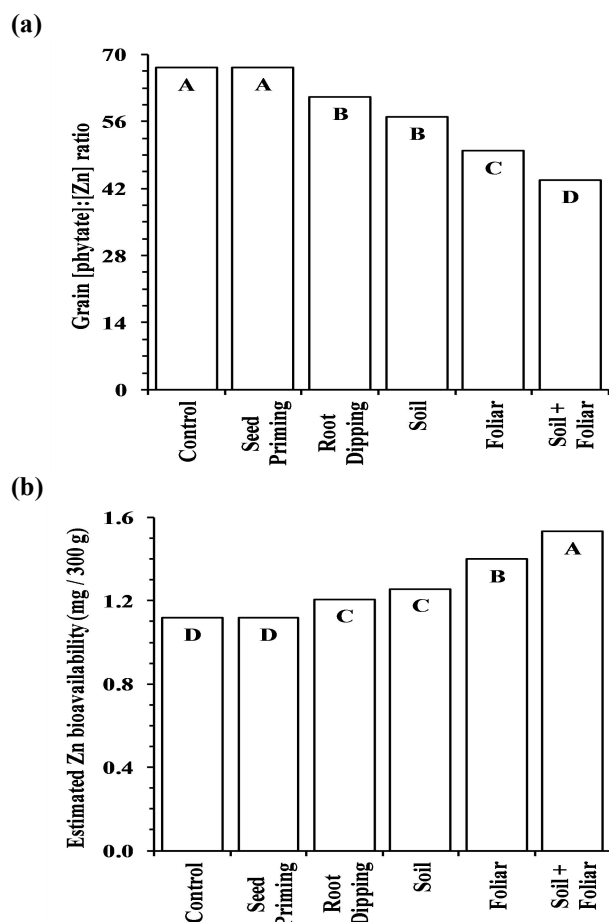
(a)

(b)

Figure 2. Zinc bioavailability estimated as [phytate]: [Zn] ratio (a) and trivariate model of Zn absorption based human Zn bioavailability (b) in grains of rice crop grown in field and differentially treated with Zn. Zinc treatments included: 0 Zn applied control (Control), priming of seeds in 0.5% w/v Zn solution (Seed priming), dipping of roots in 0.5% w/v Zn solution (Root dipping), soil application of 20 kg Zn ha⁻¹ (Soil), two sprays of 0.5% (w/v) Zn (foliar application) and combination of soil + foliar Zn applications (Soil + Foliar). Different letters in the same pan indicate significant differences by LSD at *P*≤0.05

Seed priming is cost-effective Zn treatment that increased straw and paddy yields of chickpea and wheat (Harris *et al.*, 2008); however, it did not fulfill total Zn requirement of rice plants for optimum yield (Table 2). Therefore, soil Zn application is required for optimum yields. Due to a limited Zn supply (Hamid and Ahmad, 2001) and high fixation of added Zn to soil constituents (Hussain *et al.*, 2011), optimum grain and straw yields were obtained with

combined application of Zn through soil and foliar methods (Table 2).

Increased paddy yield can partially be correlated with increased grain weight under separate and combined applications of Zn to soil and foliage (Table 2). Zinc influences various metabolic processes of healthy grain production and improvement in grain weight by Zn application was previously reported by Shivay *et al.* (2007).

Compared with 0 Zn applied control, Zn application to soil, foliage and combined soil + foliar Zn application significantly (*P*≤0.05) increased Zn concentration and content in rice grains (Fig. 1).However, maximum grain Zn concentration achieved in this study was lowerthan desired level of Zn in rice grains (35 mg Zn kg⁻¹). Foliar application of Zn at critical growth stages increased concentration and content of Zn in rice grains (Naik and Das, 2008; Stalin, 2011).Moreover, Mabesa *et al.* (2013) reported genotypic variation for increase in grain Zn concentration (1 to 10 mg Zn kg⁻¹) by foliar Zn application at heading stage of rice crop. While, increase in grain Zn content (both seed⁻¹and ha⁻¹) with the application of Zn can be related to increased grain Zn concentration, grain weight and paddy yield (Fig. 1).

Phytate is a major phosphorus storing compound in cereal grains and it acts as metal chelator in human intestine; therefore, hinders the absorption of dietary Zn and other metals into blood (Bohn *et al.*, 2008). In the current study (Table 2) and also elsewhere (Mabesa *et al.*, 2013), it was observed that soil and foliar Zn applications significantly (*P*≤0.05) reduced phytate concentration in rice grains. One reason for this is yield dilution effect as phytate content seed⁻¹ and phytate content ha⁻¹ were actually increased with Zn application (Table 3) along with increase in paddy yield and grain weight (Table 2).Decrease in grain phytate concentration with Zn application has previously been reported in cereals (Erdal *et al.*, 2002; Depar *et al.*, 2013; Hussain *et al.*, 2012b; 2013).

Desired [phytate]:[Zn] ratio in human diet for optimum Zn nutrition is <20 (Turnlund *et al.*, 1984; Weaver and Kannan 2002). Zinc application, especially soil + foliar Zn application, increased grain Zn concentration and decreased grain phytate concentration that resulted in grain [phytate]:[Zn] ratio as low as 44 (Fig. 2a). Therefore, Zn application has significant effect on grain [phytate]:[Zn] ratio but did not decrease it anywhere near to desired levels for optimum Zn bioavailability. Similarly, soil + foliar Zn application increased trivariate model of Zn absorption based estimated Zn bioavailability from 1.1 to 1.5 mg Zn for 300 g rice grain. The physiological Zn requirement of an adult human, net Zn that need to be absorbed d⁻¹, is 3 mg (Institute of Medicine, 2001) while 300 g rice biofortified grains can only ensure half of the daily Zn requirement (Fig. 2b).

Conclusion: Zinc application increased grain and straw yields; however, seed priming and root dipping had only a marginal influence on paddy yield. Combined soil + foliar Zn application was required for optimum yield from a soil low in Zn availability. Grain Zn concentration and estimated human Zn bioavailability in rice grains was maximum with the same Zn treatment. Conclusively, combined soil + foliar Zn application increased Zn bioavailability in rice grain up to medium level. However, only a limited increase in grain Zn concentration by Zn treatments suggested that molecular and genetic approaches of Zn biofortification should also be the focus of zinc biofortification programs.

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