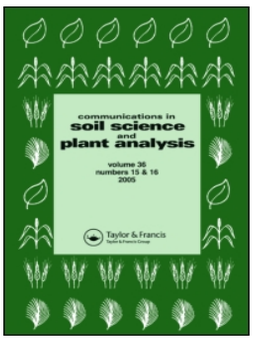

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Communications in Soil Science and Plant Analysis

ISSN: 0010-3624 (Print) 1532-2416 (Online) Journal homepage: http://www.tandfonline.com/loi/lcss20

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To cite this article: Mahalingam Govindaraj, Kedar N. Rai, Wolfgang H. Pfeiffer, Anand Kanatti & Harshad Shivade (2016) Energy-Dispersive X-ray Fluorescence Spectrometry for Cost-Effective and Rapid Screening of Pearl Millet Germplasm and Breeding Lines for Grain Iron and Zinc Density, Communications in Soil Science and Plant Analysis, 47:18, 2126-2134, DOI: 10.1080/00103624.2016.1228938

To link to this article: http://dx.doi.org/10.1080/00103624.2016.1228938

Accepted author version posted online: 27 Sep 2016.
Published online: 27 Sep 2016.

COMMUNICATIONS IN SOIL SCIENCE AND PLANT ANALYSIS
2016, VOL. 47, NO. 18, 2126–2134
http://dx.doi.org/10.1080/00103624.2016.1228938

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Energy-Dispersive X-ray Fluorescence Spectrometry for Cost-Effective and Rapid Screening of Pearl Millet Germplasm and Breeding Lines for Grain Iron and Zinc Density

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ABSTRACT

Comparison of energy-dispersive X-ray fluorescence (XRF) and inductively coupled plasma-optical emission spectroscopy (ICP) for iron (Fe) and zinc (Zn) densities in pearl millet grain samples from 11 trials showed significant differences between these two methods for both micronutrients. XRF values were more often higher than the ICP values for both micronutrients, but the differences were significant in only 15–38% genotypes for Fe and in 7–25% genotypes for Zn across the trials. In 82% genotypes the differences between these two methods were ≤6 mg kg^{-1} for Fe; and in 88% genotypes, the differences were ≤4 mg $kg^{-1}$for Zn. There were highly significant and high positive correlations between ICP and XRF for both micronutrients. Selection of genotypes above the XRF trial mean for Fe/Zn included at least 30% top-ranking genotypes based on ICP. Therefore, XRF can be used for cost-effective and rapid screening of a large number of grain samples in pearl millet biofortification programs.

ARTICLE HISTORY
Received 17 September 2015
Accepted 17 June 2016

KEYWORDS
Biofortification; iron; pearl millet; screening technique; XRF; zinc

Introduction

Pearl millet (*Pennisetum glaucum* (L.) R. Br.) is a major warm-season cereal, grown on about 28 m ha in the arid and semiarid tropical regions of Asia and Africa (Yadav et al. 2012). It is a major source of dietary energy and nutritional security to the rural households in these regions. Iron (Fe) and zinc (Zn) deficiencies have been recognized as global public health problems, affecting more than two billion people worldwide, with those in the developing countries being the most vulnerable (WHO 2002). Pearl millet has higher levels of Fe (18–121 mg kg^{-1}) and Zn (22–87 mg kg^{-1}) density than other major cereals like rice, wheat, maize, and sorghum (Dwivedi et al. 2012; Rai, Govindaraj, and Rao 2012; Rai et al. 2015b), and thus can play a significant role in reducing the nutritional gaps arising from the deficiencies of these micronutrients. For instance, it has been shown to account for 19–63% of the total Fe and 16–56% of the total Zn intake from all food sources in the pearl millet-growing areas of Maharashtra, Gujarat, and Rajasthan states of India (Parthasarathy Rao et al. 2006). It is also the cheapest source of these micronutrients as compared to other cereals and vegetables.

Crop biofortification is emerging as a highly cost-effective and sustainable agricultural approach to address micronutrient malnutrition. Considering the existence of large variability for Fe and Zn density in pearl millet (Rai, Govindaraj, and Rao 2012, 2015b, 2013; Velu et al. 2007), International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), in alliance with the HarvestPlus

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Challenge Program of the Consultative Group on International Agricultural Research (CGIAR), has undertaken a partnership-based research to further improve the levels of these micronutrients in pearl millet breeding lines and cultivars. When dealing with a large number of samples of breeding lines, reliable, rapid, and cost-effective determination of Fe and Zn density in grain samples will have a direct bearing on breeding efficiency. Atomic Absorption Spectrometry (AAS) and inductively coupled plasma optical emission spectrometry (ICP) have been widely used for the analysis of these micronutrients. However, precision techniques as they are, these methods have limitations in that they are destructive, require digestion of ground samples, which is time consuming, and have low sample throughput. These also need expensive laboratory facilities in terms of equipment, chemical reagents, and trained personnel. Therefore, these two methods do not lend themselves for cost-effective and rapid analysis of a large number of grain samples.

A recent research using AAS analysis of ground and unground grain samples of pearl millet genotype with varying grain sizes showed that the ground sample values were marginally higher than the unground sample values, though statistically significant, and there were highly significant and very high positive correlations between the values of these two sample types, for both Fe and Zn densities. Based on these results, it was suggested that ungrounded samples be used for rapid and cost-effective analysis of Fe and Zn densities in pearl millet as well as sorghum, another small-seeded crop (Sahrawat et al. 2014). Working in this direction, calibration and validation of an energy-dispersive X-ray fluorescence (XRF) method showed highly significant and very high positive correlation between XRF and ICP values, for both Fe and Zn densities in pearl millet, and thus can be used as a reliable tool for rapid and cost-effective high throughput analysis of these micronutrients (Paltridge et al. 2012). The results of this study, however, are based on a few genotypes that had a very wide range of Fe and Zn values, and only one set of genotypes with samples from unreplicated field trials was used each for calibration and validation. Hence, the objective of our research was to compare the XRF and ICP values of Fe and Zn densities for grain samples obtained from several sets of genotypes evaluated in a large number of replicated field trials, with the range in Fe and Zn values normally found in the pearl millet biofortification breeding lines.

Material and methods

Experimental materials and field trials

Seven sets of inbred lines consisting of 45–76 genotypes each and four sets of hybrids consisting of 26–40 genotypes each were evaluated in two replications in a randomized complete block design in Alfisols at Patancheru during the 2010 rainy season. The plot size in the inbred line trials was a single row of 4 m, while in the hybrid trials it was two rows of 4 m, with rows spaced 75 cm apart. Within-row plant-to-plant spacing was 10 cm. At crop maturity, open-pollinated main panicles of six to eight standing-plants (panicles without soil contact) in each plot were harvested, placed in paper bags, sundried for 10–15 days, and threshed in single-head machine thresher (Wintersteiger-129 ID780ST4, Ried, Austria). About 30 g grain samples were collected for each plot and stored for 1–2 months in clean and non-metal fold paper bags at room temperatures normally above 32°C, and then used for Fe and Zn density analysis.

Micronutrient analysis

Open-pollinated grain samples produced from each plot in all the trials were divided into two subsamples. One subsample was sent to the Waite Analytical Services Laboratory, Adelaide, Australia, for Inductively Coupled Plasma Optical Emission Spectrometer (ICP)(Thermo Fisher Scientific, Waltham MA), hereafter referred to as ICP analysis; and the other sample was analyzed at ICRISAT using energy-dispersive X-ray fluorescence spectrometry, hereafter referred to as XRF. The ICP analysis for Fe and Zn densities was done following the method described by Wheal,

Fowles, and Palmer (2011). Analysis of aluminum (Al) density as an index element for monitoring dust contamination was also performed. Grain samples were oven-dried overnight at 85 °C prior to digestion, grounded enough to pass through a 1 mm stainless steel sieve using Christie and Norris hammer mill, and stored in screw-top polycarbonate vials. The samples were digested with di-acid (nitric/perchloric acid) mixture. After digestion, the volume of the digest was made to 25 mL using distilled water, and the content was agitated for 1 min by a vortex mixer. The digests were filtered and the Fe concentration was read at 259.94 nm and the Zn concentration at 213.86 nm using ICP and these micronutrients were expressed as mg kg^{-1}. Care was taken at each step to avoid any contamination of the grains with dust particles and any other extraneous matter (Stangoulis and Sison 2008).

For XRF analysis, the calibration of Oxford Instruments X-Supreme 8000 fitted with a 10-place auto-sampler was done at Flinders University, Adelaide, Australia (Paltridge et al. 2012). For this, 20 reference pearl millet whole grain samples that had ICP-determined Fe (29–163 mg kg^{-1}) and Zn (35–100 mg kg^{-1}) densities were used to calibrate the XRF method. Thus, ICP concentrations used as reference value were entered into the machine before each sample was scanned. Clean Poly-4 film was used for each sample. According to the manufacturer, the X-Supreme 8000 scans a circle of 21 mm diameter with the sample spinner on. All scans in this study were performed in this mode, so the scanned area was 346 mm^2 (Paltridge et al. 2012). Thus, background scans fixed uniform emission toward the sampling compartment with 60 s acquisition times for each sample cup. The relationship between XRF and reference values was then established using the XRF calibrates function and a simple linear model. Calibration results showed very high correlation coefficients ($R^2 = 0.97$; $p < 0.001$) between the XRF and ICP values, for both Fe and Zn densities. To validate the calibrations, another set of pearl millet samples was randomly selected from the lines developed at ICRISAT and scanned using XRF. The validation results also showed very high correlations between XRF and the reference values ($R^2 = 0.95$; $p < 0.001$) for both micronutrients. This calibration was installed in the XRF instrument at ICRISAT for high throughput analysis of a large number of samples generated each year. Scans were conducted in Al sample cups of 30 mm diameter, 36 mm depth, and >20 g of grain weight capacity, combined with polypropylene inner cups sealed at one end with 4 μm Poly-4 XRF sample film. Cups in a batch of 10 were filled with 8–12 g of grain samples to a depth of >22 mm higher than >6 mm depth and >4 g as suggested by Paltridge et al. (2012). The cups were shaken to evenly distribute grains in the cups, which were loaded in the XRF instrument holder. It takes 13 min to complete the analysis and display the Fe and Zn densities on the monitor attached to the XRF instrument. After the analysis, the cups were removed and cleaned to prepare for the next batch of analysis.

Statistical analysis

Analysis of variance was performed following a nested design (analytical methods nested within genotypes) using the GenStat statistical package (GenStat V 14, 2011). The Pearson correlation coefficient between the two analytical methods and the least significant difference (LSD) was calculated following Gomez and Gomez (1984). The significance of correlation coefficient was tested referring to the standard table given by Snedecor and Cochran (1967). The frequency of genotypes (as percent of the total under test) was worked out for each trial and each micronutrient, where the XRF and ICP values were identical (XRF = ICP), or where the XRF values were higher than the ICP values (XRF > ICP) or the ICP values were higher than the XRF values (ICP > XRF). Where the differences between the XRF and ICP values did exist, the frequency of genotypes with statistically significant differences was also worked out using respective LSDs for various trials following Gomez and Gomez (1984).

Table 1. Mean squares for grain Fe and Zn densities in pearl millet inbred trials and hybrid trials, 2010 rainy season, Patancheru.

		Mean square							
		Fe density				Zn density			
Trial	No. Entries	Genotype	Error (a)	Method/ Genotype	Error (b)	Genotype	Error (a)	Method/ Genotype	Error (b)
Inbred trial-1 (IT 1)	70	383.8**	92.1	72.0	65.8	82.2**	36.3	27.4	21.5
Inbred trial-2 (IT 2)	45	715.1**	79.8	28.8*	16.6	201.9**	65.7	51.8	42.5
Inbred trial-3 (IT 3)	40	680.5**	59.2	31.6	24.8	171.4**	30.1	10.4	13.9
Inbred trial-4 (IT 4)	72	641.1**	54.9	29.4**	11.0	206.8**	26.5	8.6**	2.9
Inbred trial-5 (IT 5)	76	640.0**	91.9	74.0**	12.0	274.1**	38.0	19.8**	4.0
Inbred trial-6 (IT 6)	66	878.3**	50.7	22.2**	9.0	275.4**	32.9	8.0**	2.1
Inbred trial-7 (IT 7)	56	1017.8**	74.3	30.0**	9.1	305.1**	34.7	8.0**	2.3
Hybrid trial-1 (HT 1)	40	158.1**	23.0	10.0*	5.8	77.7**	15.1	5.6**	2.1
Hybrid trial-2 (HT 2)	39	231.4**	89.1	27.5**	11.0	66.7**	15.6	8.9	10.7
Hybrid trial-3 (HT 3)	28	195.2**	29.6	16.7*	7.0	75.7**	15.8	3.3*	1.7
Hybrid trial-4 (HT 4)	26	237.2**	31.1	13.6	8.2	40.1*	18.2	3.0	2.8

* and ** represent significance at 0.05 and 0.01 probability levels, respectively.

Table 2. Range and coefficient of variation (CV) for XRF and ICP values of grain Fe and Zn densities in pearl millet, 2010 rainy season, Patancheru.

	Fe density				Zn density			
	Range (mg kg^{-1})		C.V (%)		Range (mg kg^{-1})		C.V (%)	
Trial	XRF	ICP	XRF	ICP	XRF	ICP	XRF	ICP
IT-1	23–75	24–80	22.9	9.5	25–49	23–49	17.5	9.8
IT-2	33–94	29–89	12.5	9.6	24–56	23–62	23.4	8.4
IT-3	30–84	29–79	13.7	8.7	30–66	26–64	13.2	9.6
IT-4	34–102	32–98	10.6	10.0	27–61	24–60	9.4	8.7
IT-5	28–91	27–84	13.4	12.1	26–66	26–63	10.8	10.6
IT-6	30–112	30–100	11.3	9.6	28–68	25–64	10.1	10.1
IT-7	27–102	29–94	12.3	10.1	24–72	22–70	10.2	9.9
HT-1	35–67	31–63	8.6	9.0	27–46	26–45	8.2	7.9
HT-2	38–82	39–68	13.9	10.8	34–52	31–49	8.3	7.6
HT-3	38–66	36–61	9.8	7.1	30–49	29–47	8.2	7.2
HT-4	37–71	38–60	11.0	6.7	32–46	33–44	9.6	7.5

Results and discussion

The analysis of variance showed highly significant differences ($p < 0.01$) among the genotypes in all the seven inbred trials and four hybrid trials for Fe as well as Zn density ($p < 0.01$) (Table 1). Much wider differences were found for Fe density than for Zn density in all the 11 trials (Table 2). Thus, while there were three- to four-fold differences among the genotypes for Fe density, there were two- to three-fold differences for Zn density in the inbred trials, and these were consistent for XRF as well ICP methods of analysis. Similarly, while there were about two-fold differences among the genotypes for Fe density, there were 1.5-fold differences among the genotypes for Zn density in the hybrid trials.

There was no significant difference between the XRF and ICP values for Fe and Zn densities in two inbred trials (IT 1 and IT 3), and for Zn density in IT 2 (Table 1). Lack of these significant differences between the values of the two methods may be due to the larger error component in the XRF values as reflected in the higher coefficients of variation for XRF values (13.2–22.9%) than for ICP values (8.4–9.8%) in these three trials (Table 2). In the remaining four inbred trials (IT 4–IT 7), the differences between the values of the two methods were highly significant. While the XRF values were higher than the ICP values for Fe density in 55–74% of the genotypes in these trials, these differences were significant in 15–23% of the genotypes (Table 3). Similarly, while the XRF values were higher than the ICP values for Zn density in 69–88% of the genotypes in these trials, these differences were significant in 20–38% of the genotypes. In these trials, the XRF values were higher

Table 3. Percent and range of XRF and ICP values for grain Fe and Zn densities in inbred trials and hybrid trials, 2010 rainy season, Patancheru.

			XRF > ICP			ICP > XRF		
Trial	No. Entry	Trait	% of entry	Range†	XRF = ICP	% of entry	Range†	LSD (5%)
IT-1	70	Fe	54(1)*	1–19	3	41(1)*	1–18	16
		Zn	56(4)	1–12	5	37(1)	1–9	9
IT-2	45	Fe	71(9)	1–12	2	24(2)	1–11	8
		Zn	51(2)	1–14	2	44(4)	1–15	13
IT-3	40	Fe	68(8)	1–13	3	25(0)	1–10	10
		Zn	68(5)	1–11	7	15(0)	1–6	8
IT-4	72	Fe	72(15)	1–15	2	25(4)	1–11	7
		Zn	69(21)	1–10	12	14(3)	1–4	3
IT-5	76	Fe	75(20)	1–10	6	17(1)	1–10	7
		Zn	78(20)	1–8	13	5(0)	1–3	4
IT-6	66	Fe	65(15)	1–12	3	30(2)	1–7	6
		Zn	88(38)	1–7	5	5(0)	1–2	3
IT-7	56	Fe	55(23)	1–16	5	36(7)	1–9	6
		Zn	77(21)	1–8	7	11(0)	1–2	3
HT-1	40	Fe	65(13)	1–8	6	20(0)	1–3	5
		Zn	80(25)	1–6	7	3(0)	1	3
HT-2	39	Fe	85(18)	1–13	1	13(0)	1–4	7
		Zn	87(0)	1–6	2	8(0)	1–5	7
HT-3	28	Fe	79(14)	1–10	2	14(0)	1–3	5
		Zn	54(7)	1–4	3	36(7)	1–3	3
HT-4	26	Fe	69(8)	1–11	4	15(0)	1–3	6
		Zn	31(0)	1–3	10	31(8)	1–4	3

†Difference between ICP and XRF values of micronutrient densities (mg kg^{-1}).
*Values in parenthesis indicate percent of entries significant at 0.05 probability level.

than the ICP values by as much 10–16 mg kg^{-1} for Fe density and by as much as 7–10 mg kg^{-1} for Zn density in some of the genotypes. The ICP values were higher than the XRF values in 18–36% of the genotypes for Fe density, but these differences were significant in only 1–7% of the genotypes. Similarly, while the ICP values were higher than the XRF values in 3–14% of the genotypes for Zn density, these were significant in no more than 3% of the genotypes in any of the trials. The ICP values were higher than the XRF values by as much as 7–11 mg kg^{-1} for Fe density and by as much as 2–4 mg kg^{-1} for Zn density in some of the genotypes.

In hybrid trials, there were no significant differences between the XRF and ICP values for Fe and Zn densities in HT4, and for Zn density in HT2 (Table 1). Where the differences between the XRF and ICP values did exist, the XRF values for Fe density were higher than the ICP values in 65–85% of the genotypes, but these differences were significant in 8–18% genotypes only and differed by as much as 8–13 mg kg^{-1}. The XRF values were higher than the ICP values in 31–87% genotypes for Zn density, but these differences were significant in 7–25% genotypes, and this too in only two trials with the magnitude of differences being marginal (3–6 mg kg^{-1}). The ICP values were higher than the XRF values in 15–20% genotypes for Fe density. These differences, however, were no more than 4 mg kg^{-1}, and were not significant in any genotype. The XRF values were higher than the ICP values in 3–36% genotypes for Zn density. However, these differences were significant in 7–8% genotypes, which occurred in only two trials, and the differences were marginal (1–5 mg kg^{-1}).

There were highly significant and high positive correlations ($p < 0.01$) between the XRF and ICP values for Fe density in all the inbred and hybrid trials (Figure 1). Except for IT 1 where the correlation coefficient was 0.77, it varied from 0.91 to 0.97 in all the other trials. The relatively lower correlation coefficient in IT 1 could have resulted from the very high coefficient of variation (22.9%) for the XRF Fe density in this trial. If one were to select genotypes having the Fe levels above the XRF trial mean for advancing to the next stage testing, 46–57% of the genotypes in different trials would get selected, and this would include 18–28% top-ranking genotypes based on ICP values in four trials (IT 1, IT 5, IT 7, HT 1) and 32–50% of the top-ranking genotypes in seven trials (Table 4). If one to two genotypes falling below these top-ranking genotypes in IT 1,

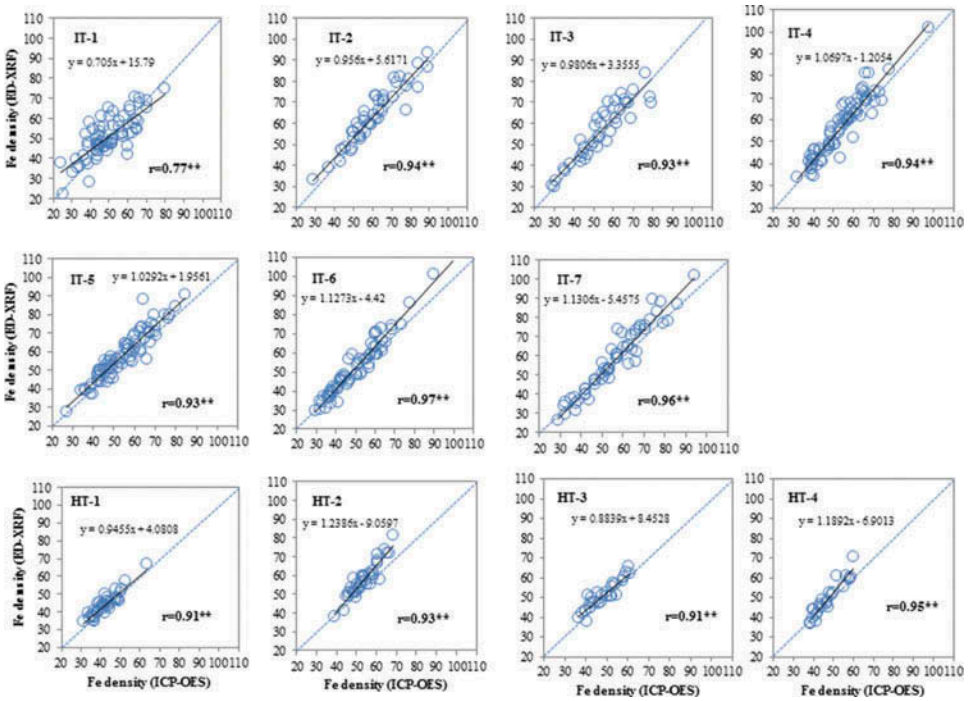

Figure 1. Correlation coefficients between XRF and ICP values for Fe density in inbred trials (IT-1 to IT-7) and hybrid trials (HT-1 to HT-4), 2010 rainy season, Patancheru. The slope trend- line between X (ICP) and Y (XRF) axes indicate slope deviation from 1:1 dash line (**, significant at $p < 0.01$).

Table 4. Comparison of XRF vs. ICP values for grain Fe and Zn densities in inbred trials and hybrid trials of pearl millet, 2010 rainy season, Patancheru.

		Fe (mg kg^{-1})					Zn (mg kg^{-1})				
Trial	No. Entry	Trial mean (XRF)	Ent≥ trial mean XRF (%)	Top entry ICP (%)	Top entry ICP (%) with few omissions	Entry omitted	Trial mean (XRF)	Ent≥ trial mean XRF (%)	Top entries ICP (%)	Top entry ICP (%) with few omissions	Entry omitted
IT-1	70	51	33 (47)*	15 (21)	23 (33)	2	38	43 (61)	11 (16)	21 (30)	4
IT-2	45	63	22 (49)	17 (38)	20 (44)	2	42	25 (56)	6(13)	17 (38)	2
IT-3	40	56	21 (53)	16 (40)	19 (48)	1	41	22 (55)	7 (18)	15 (38)	1
IT-4	72	57	39 (54)	23 (32)	36 (50)	1	43	38 (53)	22 (31)	32 (44)	2
IT-5	76	58	35 (46)	14 (18)	34 (45)	3	43	40 (53)	32 (42)	36 (47)	1
IT-6	66	53	31 (47)	26 (39)	26 (39)	–	43	29 (44)	20 (30)	28 (42)	1
IT-7	56	58	27 (48)	15 (27)	27 (48)	2	44	30 (54)	25 (45)	25 (45)	–
HT-1	40	44	22 (55)	11 (28)	17 (43)	1	37	23 (58)	19 (48)	19 (48)	–
HT-2	39	58	20 (51)	13 (33)	13 (33)	–	39	16 (41)	6 (15)	14 (36)	3
HT-3	28	51	16 (57)	14 (50)	14 (50)	–	38	15 (54)	11(39)	14 (50)	2
HT-4	26	49	12 (46)	10 (38)	10 (38)	–	37	17 (65)	14 (54)	14 (54)	–

*Values within parenthesis indicate the percent entries in each trial.

IT 5, IT 7, and HT 1 were to be discounted for, ICP-based selection would include 33–48% top-ranking genotypes in these four trials.

The correlation between the XRF and ICP values for Zn density was also highly positive and highly significant in all the inbred and hybrid trials (Figure 2). Except for IT 1 and IT 2, where the correlation coefficients were 0.63 and 0.67, respectively, it varied from 0.86 to 0.98 in the remaining nine trials. The relatively lower correlation coefficients in IT 1 and IT 2 would have resulted from the higher coefficient of variation for Zn density (17.5 and 23.4%, respectively) in these two trials. Similar to the Fe density, if one were to select genotypes exceeding the XRF trial mean for the next

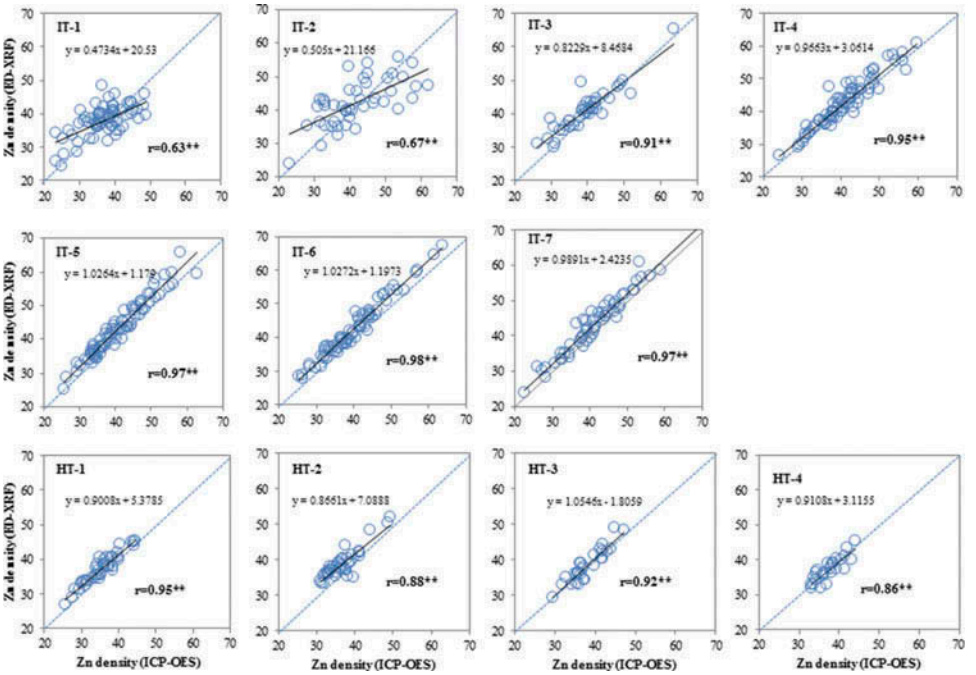

Figure 2. Correlation coefficients between XRF and ICP values for Zn density in inbred trials (IT-1 to IT-7) and hybrid trials HT-1 to HT-4, 2010 rainy season, Patancheru. The slope trend- line between X (ICP) and Y (XRF) axes indicate slope deviation from 1:1 dash line (**, significant at $p < 0.01$).

stage of evaluation of Zn density, ICP-based 13–18% top-ranking genotypes would get selected in four trials (IT 1, IT 2, IT 3, and HT 2), and 30–54% top-ranking genotypes would get selected in the remaining seven trials (Table 4). If one to four genotypes below these top-ranking genotypes in IT 1, IT 2, IT 3, and HT 2 are discounted for, then XRF-based selection above trial mean would lead to the selection of 30–38% top-ranking genotypes based on ICP values in these trials.

The results for both Fe and Zn densities in these 11 trials indicated that XRF-based selection above the trial mean would generally select about 50% of the genotypes for the next stage of testing. These genotypes would generally include no less than 15% of the top-ranking genotypes if ICP analysis were to be carried out. In fact, discounting a few genotypes, such XRF-based selection would include at least 30% of the top-ranking genotypes if the ICP analysis were to be done. Considering that there is significant genotype × environment interaction for Fe and Zn densities, and breeding lines and hybrids are sequentially evaluated in multistage testing, selection of genotypes above trial mean using XRF is unlikely to miss selecting at least 15% top-ranking genotypes based on ICP analysis at any testing stage. The effectiveness of XRF analysis can be further improved by analyzing duplicate grain samples of genotypes identified above the trial mean. This may permit safe discarding of more genotypes than those found below the trial mean in the initial analysis.

Besides selection for Fe and Zn densities as the primary traits in the biofortification breeding programs, there would also be selection for yield potential and other agronomic traits in the group of lines not discarded. Such a procedure would then discard those lines, which do not meet the desired levels of performance for these traits, thus further reducing the proportion of lines to be advanced to the next stage of inbreeding and testing. Retention of top-ranking lines for Fe and Zn densities in this group to be further advanced assumes that there is no correlation between the Fe and Zn densities on the one hand; and grain yield and agronomic traits like seed size, flowering time, and plant height on the other. Pearl millet studies show that there is either no correlation of Fe and Zn densities with grain yield, or there is negative but low correlation, which is not always significant

(Gupta et al. 2009; Kanatti et al. 2014; Rai, Govindaraj, and Rao 2012). These studies also suggest that the correlation of Fe and Zn with grain size is either non-significant or there is significant positive correlation. Thus, lines selected for high Fe and Zn densities can also be in high-yielding and in large-seeded backgrounds.

Considering that the XRF values are generally higher than the ICP values, for both Fe and Zn densities, it is to be viewed as a simple technique for rapid and cost-effective discarding of genotypes with low density of these micronutrients. One concern about this method has been raised that XRF, unlike ICP, does not lend for the analysis of dust contamination indicators such as aluminum (Al) and titanium (Ti). Following simple yet careful grain sampling protocols, it has been observed that dust contamination is not a significant factor influencing Fe density in pearl millet (Rai et al. 2015a), and Zn density is not influenced by dust contamination (Paltridge et al. 2012). Earlier studies in cereals showed that Al density exceeding 5 mg kg^{-1} is frequently associated with dust contamination, which leads to overestimates of Fe density (Pfeiffer and McClafferty 2007). In the present study, Al density was no more than 3 mg kg^{-1} in 97% of the grain samples. Further, it is unlikely that if a genotype registers high Fe density due to dust contamination, the same genotypes or the progenies derived from them will also have high Fe density on account of dust contamination at the subsequent stages of testing. At the final testing stage when there would be much fewer genotypes to be analyzed to select those for use in cultivar development or for use in crossing and population development, Fe and Zn densities should be analyzed using ICP for more accurate determination of the densities of these micronutrients.

Funding

This research, supported by HarvestPlus Biofortification Program of the CGIAR, was conducted under CRP Agriculture for Nutrition and Health.

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