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Genetic enhancement of grain iron and zinc content in pearl millet

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Abstract

Pearl millet (*Pennisetum glaucum* (L.) R. Br.) is a highly nutritious warm season cereal. In a major initiative to further improve its nutritive value, a partnership-based research has been under way at ICRISAT to eventually develop high-yielding hybrids with higher levels of iron (Fe) and zinc (Zn) contents. Investigations related to factors that can enhance breeding efficiency showed large variability for and high levels of both Fe and Zn content in breeding lines and populations (>80 ppm Fe and >60 ppm Zn). The Fe and Zn contents were positively and highly significantly correlated (r=0.49 to 0.71), implying the possibility of simultaneous effective selection for both micronutrients. There were negative correlations between these micronutrients and grain yield, though significant only in the case of Fe content and in only three of the six trials (r= -0.39 to -0.58), indicating that selection for high Fe and Zn content may be possible without significant compromise on grain yield. These micronutrients were not correlated with seed size and time to flower. Largely additive genetic control with the predictability ratio of 0.91 for both Fe and Zn content indicated that to breed micronutrient-dense hybrids would require breeding both parental lines with higher levels of these micronutrients.

Keywords: correlation, genetics, germplasm, micronutrients, pearl millet, variability, yield

Introduction

Micronutrient malnutrition arising from dietary deficiency of one or more essential micronutrients affects two-third of world’s population (White & Broadley, 2009; Stein, 2010). The mineral elements most commonly lacking in human diets are iron (Fe) and zinc (Zn), which rank fifth and sixth, respectively, among the top ten risk factors contributing to burden of disease, especially in the developing countries (WHO, 2002). Crop biofortification is a sustainable and cost-effective approach to address micronutrient malnutrition, especially in the developing world (Stein *et al.,* 2007; Bouis *et al.*, 2011). It has the potential to help alleviate the suffering, death, disability, and failures to achieve human potential, which result from micronutrient deficiency-related diseases. In comparison to other strategies (fortification, supplementation or dietary diversification), it provides a truly feasible means of reaching out to remote and rural areas to deliver naturally-fortified foods to population groups with limited access to diverse diets, supplements and commercially fortified foods (Bouis *et al.*, 2011). Moreover, as the trace mineral requirements in human and plant nutrition are similar, biofortification could improve human nutrition as well as farm productivity (Ma, 2007).

Pearl millet [*Pennisetum glaucum* (L.) R. Br.] is a major warm-season cereal grown on more than 27 million ha in some of the harshest environments in the arid and semi-arid tropical regions of Africa (17 million ha) and Asia (10 million ha), with India being the largest producer, cultivating this crop on about 9 million ha. In these regions, pearl millet is a staple food of more than 90 million people. Pearl millet is a highly nutritious cereal with high levels of metabolizable energy and protein, and more balanced amino acid profile (Andrews & Kumar, 1992). Processing technologies for preparing various types of alternative and health food products have been developed. These products have been shown to have lower glycemic index than similar products produced from wheat (Sehgal *et al*., 2004), thus increasing the food value of pearl millet for those prone to diabetics. Pearl millet grains lack gluten, unlike most of the major cereals, thus enhancing its health value for those allergic to gluten (Dahlberg *et al*., 2004). Pearl millet is less prone to aflatoxin contamination than say sorghum and maize. It has been reported that eggs produced from layers fed on pearl millet-based diets have lower levels of low-density lipoprotein (Collins *et al*., 1997), thus providing for production of designer eggs for those with high cholesterol. These findings, not widely known, suggest that pearl millet can play an important role not only towards contributing to the food and nutritional security of the poor in the pearl millet growing areas of India and sub-Saharan Africa, but could also have potential health value for the affluent.

A preliminary study conducted with a limited number of 27 genotypes at ICRISAT had shown high levels of and large variability for both iron (40 to 580 ppm) and zinc content (10 to 66 ppm) in pearl millet grains (Jambunathan & Subramanian, 1988). These were much higher than those in other cereals. Pearl millet is a significant source of these micronutrients both in India and sub-Saharan Africa. For instance, it accounts for 15-63% of the total cereal consumption in some of the major pearl millet growing states of India such as Maharashtra, Gujarat and Rajasthan; and it accounts for 19-63% of the Fe and 16-56% of the Zn intake from all food sources (Parthasarathy Rao *et al*., 2006). It is also the cheapest source of these micronutrients as compared to other cereals and vegetables.

Considering the high nutritional value of pearl millet and the potential contribution it can make to improving the nutrition of those heavily dependent on it as a major food crop, ICRISAT, supported by the HarvestPlus Challenge Program of the CGIAR, initiated a research program to breed improved breeding lines and hybrid parents with elevated levels of iron (Fe), zinc (Zn) for eventual use by its partners to develop high-yielding hybrids with higher levels of these micronutrients. Much of the work done so far has dealt with the factors that have a direct bearing on enhanced breeding efficiency. In this paper we report the results of studies related to the magnitude of variability for Fe and Zn content, character association, and genetics of these micronutrients.

Materials and methods

Micronutrient variability trials

The variability for grain Fe and Zn content was evaluated in two sets of breeding lines planted in randomized complete block design with two replications in Alfisols at Patancheru during the 2011 rainy season. Each plot consisted of 1-row of 4 m length with 75 cm spacing between the rows and 10 cm spacing within the rows. Normal crop management practices were followed with 80 kg/ha of applied N and 40 kg/ha of P_2O_5. Set I consisted of 386 advanced breeding lines (mostly F_7 onwards) from the conventional hybrid parents breeding program where Fe and Zn are not the selection criteria. Set II consisted of 232 early-generation progenies (S_1-S_3) derived from seven populations that had earlier been identified as sources of high Fe and Zn contents.

Character association trials

Four Initial Hybrid trials (IHTs) and two Advance Hybrid Trials (AHTs) were conducted in Alfisols at Patancheru. Planting and crop management procedures were same as for the micronutrient variability trials. All four IHTs were planted in 2-row plots and both AHTs in 4-row plots of 4 m length, replicated three times in randomized complete block design. Panicles harvested at maturity were harvested, threshed, dried, and grain yield recorded. Random samples of 200 grains were used to estimate 1000-seed weight. Plots were visually assessed for days to 50% flowering when main panicles of 50% of the plants in the plot had fully emerged stigmas.

Line x tester trial

This trial consisted of 72 hybrids developed from crosses between 8 lines and 9 testers. These were planted in 1-row plots of 2 m length, replicated two times in a randomized complete block design. The 17 inbred lines involved in these hybrids were also planted by the side of the hybrid trial in the same design and same plot size and replications. These trials were conducted during the 2009 summer season as a part of a Ph.D. research. Planting and crop management practices for these two trials were similar to those mentioned for the micronutrient variability trial except that there was 60 cm spacing between the rows in both trial.

Micronutrient analysis

Grain samples collected from all the above experiments were sun dried for 7-10 days and stored for 1-2 months at room temperatures before laboratory analysis of Fe and Zn contents. Until 2010 most of these analyses were done using inductively coupled plasma optical emission spectroscopy (ICP-OES) method (Wheal *et al.*, 2011) at the Waite Analytical Services laboratory, Adelaide, Australia. Thus the grain samples of the combining ability trial conducted in 2009 were analyzed using ICP-OES. Although highly precise, this method is costly (about US$ 18/sample), analyzes no more than 200 samples/day, and involves a month of the data turn over time. In search for a rapid and cost-effective screening technique, near-infrared reflectance spectrometer (NIRS) at the International Livestock Research Institute laboratory, Patancheru was evaluated for its effectiveness using grain samples from eight trials (a designated B-line trial of 98 entries, 3 population progeny trials of 146-260 entries, and 4 hybrid trials of 32-44 entries) which had already been analyzed for Fe and Zn content using ICP-OES technique. The grain samples of these 8 trials were also analyzed at ICRISAT using an energy-dispersive X-ray fluorescence Spectrometry (EDXRF) method that had been standardized at the Flinders University, Australia (Paltridge *et al.*, 2012)

Statistical analysis

Fixed model analysis of variance of all the trials and correlations were done following Gomez & Gomez, 1984). Line x tester analysis of combining ability was done following Kempthorne (1957) and Arunachalam (1974).

Results and discussion

Large variability was observed both for iron (Fe) and zinc (Zn) content, with Fe ranging from 18 to 97ppm and Zn varying from 22 to 69 ppm in the advance breeding lines; and Fe ranging from 52 to 135 ppm and Zn ranging from 40 to 92 ppm in the population progenies (data not presented). While 72% of the advance breeding lines had <60 ppm Fe, 70% of the population progenies had >80ppm Fe with 20% of these exceeding 100 ppm level (Table 1). A similar pattern was observed for Zn content with 48% of the breeding lines having $\leq$40 ppm Zn, and 50% of the population progenies having >60 ppm content. This was not unexpected as, unlike most of the advance breeding lines, the progenies were all derived from seven diverse population (including open-pollinated varieties released in India) that are largely or entirely based on *iniari* germplasm. This germplasm in an earlier study was found as the most promising source of both high Fe and Zn contents (Velu *et al.,* 2007). These results suggest that while 61-80 ppm Fe and 41-60 ppm can be found in a significant number of breeding lines in elite genetic backgrounds for their direct use in hybrid parents development, population progenies with much higher levels are available as donor sources for genetic enhancement of these micronutrients.

Consistently positive and highly significant correlations between Fe and Zn density ($r = 0.49$ to 0.71; $P< 0.01$) were observed in all the six trials (Table 2). Earlier pearl millet studies have also shown highly significant and positive correlations between the Fe and Zn contents (Velu *et al*., 2007, 2008; Gupta *et al*., 2009). Positive correlations between these two minerals have also been reported in maize (Maziya-Dixon *et al*., 2000, Long *et al*., 2004), sorghum (Reddy *et al*., 2005; Ashok Kumar *et al*., 2009), wheat (Monasterio & Graham, 2000; Morgounov *et al*., 2007; Peleg *et al*., 2008; Velu *et al*., 2011a), and rice (Doesthale *et al*., 1979; Kabir *et al*., 2003). These results would imply that simultaneous selection for high levels of both Fe and Zn contents are possible. While there was no correlation between the Zn content and grain yield, there were significant negative correlations between the Fe content and grain yield in three of the six trials (r = -0.39 to -0.58). An earlier pearl millet study also showed that neither Fe nor Zn content was correlated with grain yield (Gupta *et al.*, 2009). Studies in other cereals such as sorghum (Reddy *et al.*, 2005, Ashok Kumar *et al.*, 2007), wheat (Vogel *et al.*, 1989; Morgounov *et al.*, 2007) showed both micronutrients to be significantly negatively correlated with grain yield. Chakraborti *et al.* (2009) observed significant negative correlation between Fe content and grain yield but no correlation between Zn content and grain yield in maize. Neither Fe content nor Zn content were associated with seed size and time to flower in the present study, supporting the results of earlier pearl millet studies (Velu *et al.*, 2008; Gupta *et al.*, 2009). These results indicate that both Fe and Zn contents in pearl millet can be genetically enhanced without compromising on grain yield, large seed size and earliness.

In the line x tester trial, there were highly significant differences ($P<0.01$) among the parents and among the hybrids, for both Fe and Zn contents (Table 3). While the main effects due to lines as well as due to testers were highly significant for both micronutrients, the line x tester interaction was non-significant for Fe content, and though significant for Zn content, it accounted for much less of the variability as compared to the main effects. This clearly showed up in the variance due to general combining ability (σ^2gca) as the more predominant component and predictability ratio closer to unity, implying a larger role of additive gene action in controlling both micronutrients. An earlier pearl millet study also reported largely additive gene action for these two micronutrients (Velu *et al.*, 2011b). This nature of gene action was further supported by highly significant and positive correlation between the mid-parental values and hybrid performance for both micronutrients ((r = 0.70 for Fe and 0.80 for Zn) (Figure 1). This would imply that to breed hybrids with high levels of Fe and Zn content would require incorporating these traits in both parental lines of the hybrids. It was also observed that there was highly significant and positive correlation between the performance *per se* of the lines and their general combining ability for both micronutrients (r= 0.77 for Fe and 0.86 for Zn), implying that based on the performance of the lines *per se*, about 40-50% of the poor general combiners can be discarded without testing for their general combing ability (Figure 2), and thus saving on the resources otherwise required for general combining ability evaluation. Largely additive gene action for Fe and Zn has also been reported in maize (Brkic *et al*., 2003; Long *et al*., 2004) and rice (Zhang *et al*., 2004). However, another study on maize (Chakraborti *et al*., 2010) reported largely additive gene action for Fe content, but largely non-additive gene action for Zn content.

In search for rapid and cost-effective methods for mineral analysis, Perls Prussian Blue staining, a calorimetric method, was standardized for Fe content (Velu *et al.*, 2006) and Dithizone (DTZ) staining for Zn content (Velu *et al*., 2008) in pearl millet. While both methods were effective in discarding lines with low mineral contents, they could only categorize lines into low, medium and high mineral content groups, and were not able to clearly distinguish the lines with average mineral content from those having high content of these micronutrients. Evaluation of NIRS technique using grain samples from 8 trials that had previously been analyzed for the Fe and Zn content using ICP-OES method, showed that it generally overestimated the Fe content by 26 to 55 % as compared to the ICP-OES method (reference precision analysis) (data not presented). A similar pattern of overestimation was observed for Zn content. The correlation between the ICP-OES values and NIRS values were highly significant, ranging from 0.34 to 0.75 for Fe and 0.24 to 0.58 for Zn content (Table 4). The DE-XRF method overestimated Fe content by 4 to 9% in four trials, underestimated it by 2 to 4% in three trials, and had similar Fe value as for the ICP-OES method in one trial. A similar pattern was found for the Zn content. The correlations between the ICP-OES and ED-XRF values were highly significant both for Fe and Zn content (r =0.90 to 0.95 for Fe content and 0.88 to 0.98 .for the Zn content). In the validation study of pearl millet grain samples Paltridge et al. (2012) found very high correlations between the ICP-OES and ED-XRF methods, both for Fe content (r= 0.97) and Zn content (r = 0.98). In comparison to the ICP-OES method, the ED-XRF method costs much less (US$ 2/day), enables the analysis of large number of samples (300 samples/day), and has high degree of precision, which is good enough for application in a genetic enhancement program.

Conclusions

Pearl millet biofortification research reported in this paper showed large variability for and high levels of both grain iron and zinc contents. Lines identified for high levels of these micronutrients need to be further studied for their stability across environments. Zinc content was not associated with grain yield and grain size, while iron content was negatively associated with grain yield, but only in 50% of the trials. Therefore, this subject merits further study as it has a direct bearing on the effectiveness of simultaneous selections for higher grain yield and higher level of iron content. Predominantly additive gene action for both Fe and Zn contents implies that to breed hybrids with higher levels of iron and zinc would entail breeding these traits into both parental lines, effectiveness of which can be enhanced with the application of marker-assisted selection and gene deployment. Consistently positive and highly significant correlation between the Fe and Zn contents showed that simultaneous selection for both micronutrients will be highly effective.   The ED-XRF extensively validated and found as a rapid and cost effective screening technique with high level of precision can be effectively used to enhance the breeding efficiency for both micronutrients

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Table 1 Variability for grain iron (Fe) and zinc (Zn) content in pearl millet, 2011 rainy season, Patancheru

Material	No of entries	Percent entries in micronutrient (ppm) class					
		≤ 40	41- 60	61- 80	81-100	101-120	≥ 121
		Iron content					
Advance breeding lines	386	25	47	23	5	0	0
Population progenies	232	0	2	28	50	17	3
		Zinc content					
Advance breeding lines	386	48	49	3	0	0	0
Population progenies	232	0	50	46	4	0	0

Table 2 Correlation among grain iron (Fe) and zinc (Zn) content, grain yield (GY), 1000-seed weight (SW) and days to 50% flower (DTF) in four Initial Hybrid Trials (IHTs) and two Advance Hybrid Trials (AHTs) of pearl millet, 2011 rainy season, Patancheru

Trial	No. of entries	Correlation coefficient (r) between						
		Fe vs. Zn	Fe vs. GY	Zn vs. GY	Fe vs. SW	Zn vs. SW	Fe vs. DTF	Zn vs. DTF
AHT-A	24	0.53**	-0.13	0.17	-0.01	0.05	-0.05	0.34
AHT-B	28	0.49**	-0.24	-0.32	0.21	0.02	-0.59**	-0.22
IHT-A1	30	0.67**	-0.58**	-0.24	0.32	0.22	-0.16	0.19
IHT-A2	26	0.67**	-0.39*	-0.04	0.12	-0.05	-0.20	0.08
IHT-B1	36	0.71**	-0.27	-0.18	0.04	0.29	-0.26	-0.09
IHT-B2	34	0.58**	-0.48**	-0.28	-0.15	0.07	-0.26	-0.18

*, **, Significant at P ≤0.05 and P ≤0.01 probability level, respectively.

Table 3 Analysis of variance for combining ability for grain iron (Fe) and zinc (Zn) content in line × tester trial, 2009 summer season, Patancheru

Source of variation	df	Mean square	
		Fe (ppm)	Zn (ppm)
		Parental trial	
Replication	1	368.9	0.3
Parents	16	1340.6**	632.5**
Error	16	113.6	26.7
		Hybrid trial	
Replication	1	0.1	2.8
Crosses	71	288.4**	218.8**
Lines	7	990.9**	771.3**
Testers	8	1132.8**	938.0**
Lines × Testers	56	80.0	47.0*
Error	71	55.6	26.9
σ^2 gca		60.2	49.7
σ^2 sca		12.2	10.0
Predictability ratio		0.91	0.91

*, **, Significant at 0.05 and 0.01 0probability levels, respectively.

Table 4 Correlation among energy-dispersion X-ray fluorescence (EDXRF), inductively coupled plasma optical emmission spectrometer (ICP-OES) and near-infrared reflectance spectrometer (NIRS) estimates for grain iron (Fe) and zinc (Zn) content in various trials of pearl millet, Patancheru

Material	Season/ year	No. of entries	Correlation coefficient (r) between			
			ICP-OES vs. EDXRF		ICP-OES vs. NIRS	
			Fe	Zn	Fe	Zn
Designated B-lines	Summer 2009	98	0.95**	0.98**	0.75**	0.52**
ICMV 221 S_1 progenies	Summer 2009	146	0.90**	0.93**	0.53**	0.35**
AIMP 92901 S_1 progenies	Rainy 2009	215	0.92**	0.95**	0.41**	0.41**
ICMR 312 S_1 progenies	Rainy 2009	260	0.91**	0.92**	0.34**	0.40**
Hybrid trial-1	Rainy 2010	44	0.94**	0.89**	0.58**	0.48**
Hybrid trial-2	Rainy 2010	40	0.93**	0.96**	0.49**	0.43**
Hybrid trial-3	Rainy 2010	32	0.91**	0.92**	0.50**	0.43*
Hybrid trial-4	Rainy 2010	34	0.94**	0.88**	0.52**	0.24

*, **, Significant at P ≤0.05 and P ≤0.01 probability level, respectively.

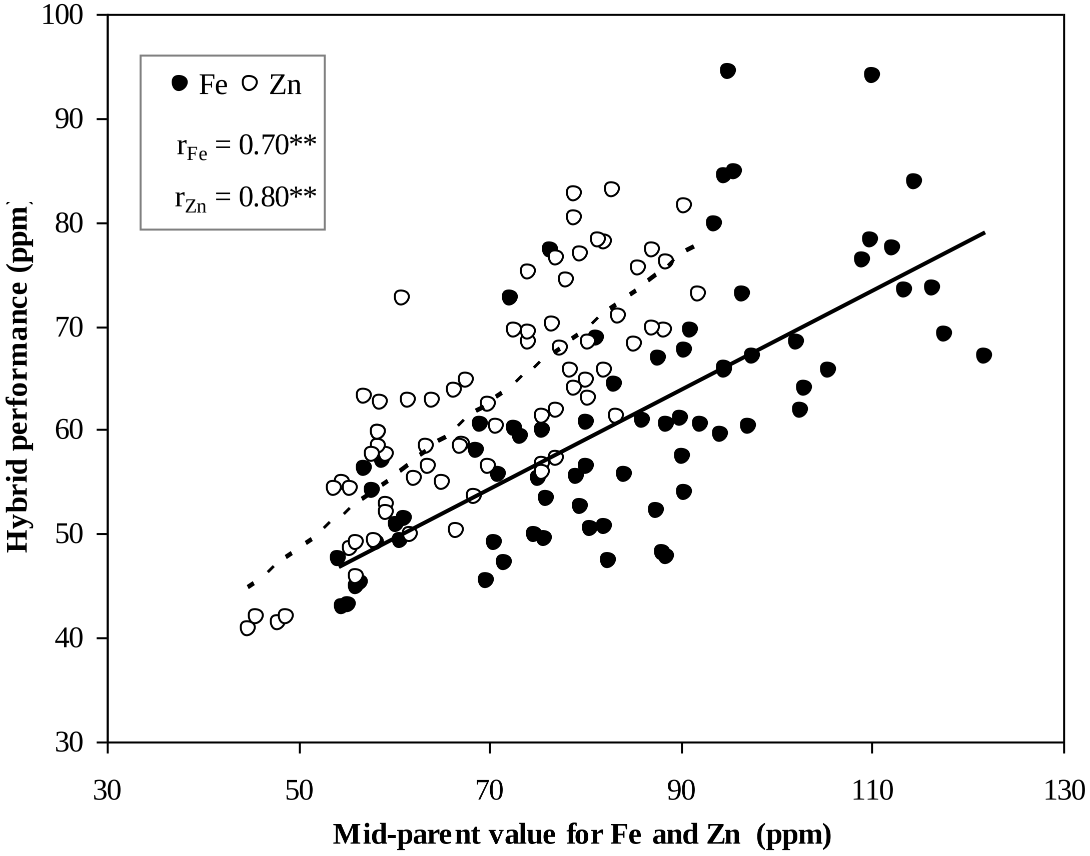

Figure 1 Relationship between mid-parent values and hybrid performance for grain iron (Fe) and zinc (Zn) content in line × tester trial, 2009 summer season, Patancheru.

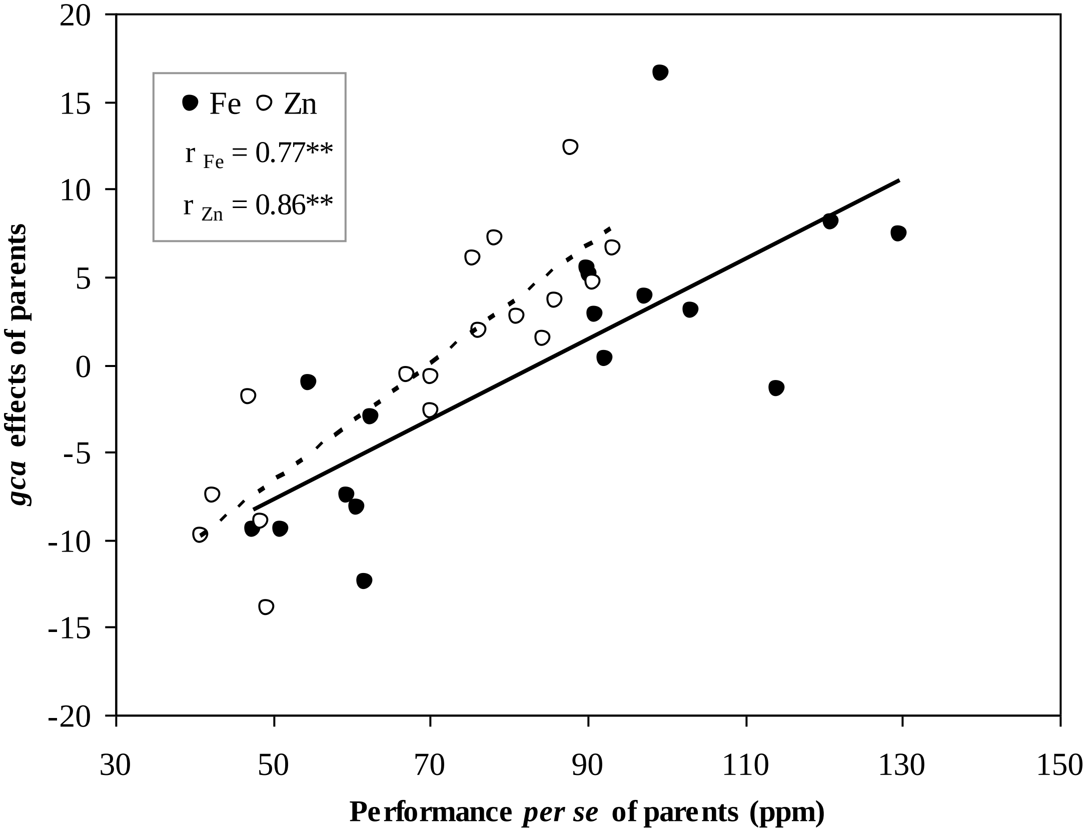

Figure 2 Relationship between performance *per se* of parents and their *gca* effects for grain iron (Fe) and zinc (Zn) content in line × tester trial, 2009 summer season, Patancheru.