A simple and rapid screening method for grain zinc content in pearl millet

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Introduction

Malnutrition arising from dietary deficiency of critically important mineral micronutrients such as iron (Fe) and zinc (Zn) is a serious health problem affecting nearly half of the world's population (WHO 2002). An estimated 49% of world population is at risk due to low intake of Zn (International Zinc Association 2000). The problem is especially serious and widespread in developing and under-developed countries of Africa and Asia, where cereal- and legume-based foods dominate in the diet of people (Welch 2002). A preliminary research strategy has shown that among staple food crops, pearl millet (*Pennisetum glaucum*) is the cheapest source of Zn (Parthasarathy Rao et al. 2006). Biofortified (Zn-enriched) pearl millet will therefore, serve as a low-cost solution in combating Zn malnutrition for people heavily dependent on this crop.

Pearl millet, grown on about 26 million ha in the arid and semi-arid regions of Asia and Africa, serves as a major source of dietary energy for millions of people living in these countries. Over twofold variation has been found for grain Zn content (24.5–64.8 mg kg^{-1}) in improved populations and breeding lines of this crop (Velu et al. 2007), indicating the existence of large genetic variability for the improvement of this trait and hence the scope for effective selection. However, the major constraint in its genetic enhancement is the high cost involved in the estimation of grain Zn content. Presently, Zn estimation is carried out by digesting the grain samples with di-acid (nitric/perchloric acid) or tri-acid mixture and determining Zn content in the digest using atomic absorption spectrophotometry (AAS) or inductively coupled plasma atomic-emission spectrophotometry (ICP-AES) (Sahrawat et al. 2002). These laboratory methods not only require expensive instrumentation, but are also time-consuming with fairly high cost involved in screening a large number of grain samples produced in the breeding program. A rapid screening method using Perl's Prussian blue stain has been standardized for pearl millet that serves as a qualitative selection criterion for grain Fe content (Velu et al. 2006). This method has been found efficient in at least discarding lines with low Fe content while screening a large number of germplasm or breeding lines. Similarly, Dithizone (DTZ: 1,5-diphenyl thiocarbazone), a Zn-chelating agent has been recommended for use in staining to locate Zn in different organisms, such as algae (Pawlik-Skowronska 2003), yeast (Bilinski and Miller 1983) and salmon (Paulsen et al. 2001). This technique has also been used in maize (*Zea mays*) and wheat (*Triticum aestivum*) seeds. A recent study on wheat showed that DTZ staining method could be used as a rapid, semi-quantitative method to estimate Zn concentrations of flour and screen the genotypes for Zn concentrations in seeds (Ozturk et al. 2006). The objective of the present study was to evaluate the effectiveness of this method for screening pearl millet grain samples for Zn.

Materials and methods

Experimental materials. A 29-entries trial representing a wide range of grain Zn content (14 high, 8 medium, 6 low lines along with a check, WC-C 75) was evaluated in field experiments in five different seasons for genotype × environment interaction at the International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru, India. The estimation of grain Zn content was done using the sib-mated grain samples from 2005 rainy and 2006 summer seasons following the AAS method (Jorhem 1993) at the National Institute of Nutrition (NIN), Hyderabad, India. Of these, nine pearl millet genotypes (7 B-lines and 2 partial inbreds) with a wide range of grain Zn content (29.2–71.2 mg kg^{-1}) were chosen to standardize the DTZ staining procedure. The procedure was further validated using the grain samples of the remaining 20 entries obtained from sib-mating in a twice replicated trial conducted in Alfisols at Patancheru during the 2005 rainy and 2006 summer seasons. Extra care was taken to avoid physical contamination of grains

with dust and extraneous matter in the field and during threshing.

Reagents. In wheat, DTZ staining was standardized to assess the concentration of Zn in the whole and germinating seeds and flour samples (Ozturk et al. 2006) by using DTZ and methanol reagents. In the present study, this protocol was standardized by using DTZ from Sigma Aldrich (99%) company and analytical grade pure methanol from Merck, India. The DTZ solution was freshly prepared by dissolving 300 mg of DTZ in 1 L of analytical grade pure methanol (ie, 300 mg L^{-1}). In wheat, a concentration of 500 mg L^{-1} has been recommended. However, to determine the optimum concentration required for our study, we used a range of DTZ concentrations (100, 200, 250, 300, 350, 400, 450 and 500 mg L^{-1}) in analytical grade pure methanol for staining flour samples of high, medium and low Zn levels. We found that the intensity of color did not differ with concentrations from 300 to 500 mg L^{-1}. However, the intensity of red-purple color of the solution in the high Zn lines was less at DTZ concentrations between 100 and 250 mg L^{-1}. Hence, 300 mg L^{-1} was considered as the optimum concentration for a reliable classification of lines in broad Zn concentration classes.

Staining procedure. Whole grain flour samples were obtained by grinding dry pearl millet seeds in Udy cyclone sample mill (Udy Corporation, USA). One gram of flour sample was placed in Borosilicate glass test tubes. The cyclone sample mill was cleaned adequately after grinding each sample to avoid cross contamination. Samples were stained with 5 ml of freshly prepared DTZ solution (300 mg L^{-1} in analytical grade pure methanol) and thoroughly mixed by vigorous shaking using Vortex® apparatus for 2 min. The samples were then incubated for 15 min for maximum color development. The intensity of color was visually scored on a 1–3 scale, where 1 = less intense red color; 2 = medium red color; and 3 = more intense red color. The intensity of red color in the flour was compared with the levels of grain Zn content determined in digests using AAS for the respective genotypes. The red color produced by DTZ staining was stable at least for 2 h. Fading of the color was evident only after 3 h.

Results and discussion

Whole grain flour of pearl millet genotypes with different concentrations of Zn content, when treated with DTZ stain showed an increase in the intensity of red color formation with increasing Zn concentration in the flour. The intensity of red color varied in genotypes having low (n=2), medium (n=3) and high (n=4) grain Zn content. In genotypes having a high Zn content (50–71 mg kg^{-1}), the red color was more intense than in those having medium Zn content (39–47 mg kg^{-1}) (Table 1). The intensity of red color was very less or none in genotypes having less than 37 mg kg^{-1} Zn content (Fig. 1). DTZ staining can, therefore, be useful, at least in separating flour samples with Zn concentrations lower than 40 mg kg^{-1}. These results suggest that DTZ staining is effective in separating genotypes with high and low Zn content. The relationship between grain Zn content and intensity of red color produced by the Zn-DTZ complex was quite strong, indicating that the higher the grain Zn content, the greater will be the intensity of red color. The dark-reddish patches on the surface of flour samples in Figure 1 may be pieces of Zn-rich embryo and aleurone that could not be ground very finely or they are related to the color of the seed (gray colored seeds).

Table 1. DTZ staining reaction and grain Zn content in nine pearl millet genotypes tested at ICRISAT, Patancheru, India.

		Zn content (mg kg^{-1})			Stain score	
Entry	Class	Rainy 2005	Summer 2006	Mean	Rainy 2005	Summer 2006
AIMP 92901 S1-15-1-2-B	High	64	71	68	3	3
843 B	High	51	70	61	3	3
863 B	High	56	50	53	3	3
ICMB 98222	High	53	57	55	3	3
ICMB 88004	Medium	45	47	46	2	2
ICMB 00888	Medium	40	45	43	2	2
MC 94 C2-S1-46-1-1-B	Medium	39	42	41	2	2
ICMB 90111	Low	26	37	32	1	1
81 B	Low	29	29	29	1	1
Mean		45	50	48	–	–
SE±		4.2	4.7	4.3	–	–

The standardized Zn-staining protocol was further validated using 20 diverse genotypes (2 B-lines, 8 partial inbreds and 10 improved populations) having a wide range in grain Zn content (27.7–64.6 mg kg^{-1}) (Table 2). Among the 20 entries, six entries with high Zn (50–65 mg kg^{-1}) produced deep-red color, 9 entries with medium Zn (38–49 mg kg^{-1}) and a check variety WC-C 75 produced medium-dense red color and four entries with low Zn (<37 mg kg^{-1}) showed no color or less intensity color development.

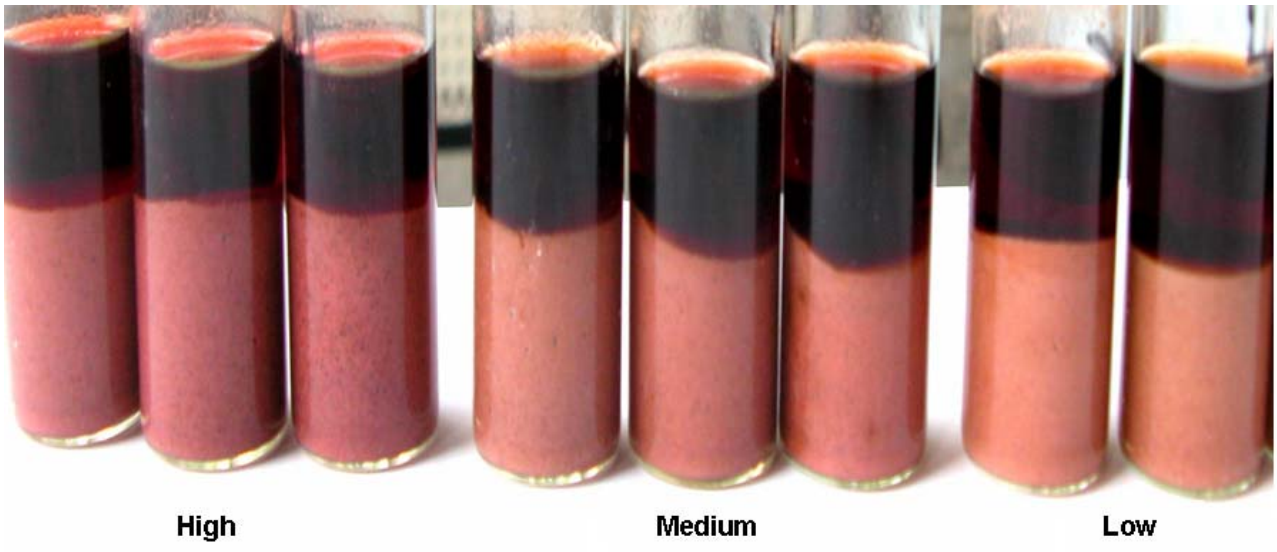

Figure 1. The intensity of red color in high (H), medium (M) and low (L) Zn content entries with differential DTZ staining reaction.

Table 2. DTZ staining reaction and grain Zn content in 20 pearl millet genotypes tested at ICRISAT Patancheru, India.

		Zn content (mg kg^{-1})			Stain score	
Entry	Class	Rainy 2005	Summer 2006	Mean	Rainy 2005	Summer 2006
ICMB 00999	High	57	61	59	3	3
IPC 843	High	54	61	58	3	3
GB 8735	High	51	65	58	3	3
EEBC	High	54	60	57	3	3
ICMS 7704-S1-51-4-1-1-B	High	50	56	53	3	3
AIMP 92901 S1-183-2-2-B	High	50	52	51	3	3
ICMB 94111	Medium	46	48	47	2	2
ICMV 93074 S1-9-1-1-1-B	Medium	40	49	45	2	2
NCD_2 Bulk	Medium	40	49	45	2	2
ICMV 91059 S1-58-2-2-2-B	Medium	42	46	44	2	2
CZI 96-21	Medium	42	45	44	2	2
Jakharana pop	Medium	42	45	44	2	2
SDMV 93032-S1-93-3-2-2	Medium	39	47	43	2	2
CZI-98-11	Medium	39	46	43	2	2
SDMV 90031-S1-84-1-1-2-B	Medium	41	43	42	2	2
HTP 94/54	Low	32	37	35	1	1
SOSAT C88	Low	29	36	33	1	1
SDMV 90031-S1-93-3-1-1-B	Low	33	31	32	1	1
ICMS 8511 S1-17-2-1-1-B	Low	23	29	26	1	1
WC-C 75 (check)	Medium	38	45	42	2	2
Mean		42	47	43	–	–
SE±		1.9	2.5	2.0	–	–
CV (%)		11	15	12	–	–

The intensity of red color served as a reliable and efficient semi-qualitative selection criterion for grain Zn in pearl millet, for separating genotypes with high grain Zn from those with low grain Zn. This simple staining method may therefore be used in rapid screening of a large number of pearl millet genotypes (germplasm accessions or advanced progenies or breeding lines) for grain Zn content. This method can be carried out at room temperature with no costly and specific equipment required other than a grinder (cyclone mill) and simple glassware. Using a cyclone sample mill, a technician can analyze about 120–150 samples per day. On the other hand, estimation of grain Zn content with AAS or ICP involves higher cost, ranging between US$ 5 to US$ 10 in various laboratories (excluding shipping cost of the samples). In addition, these procedures (AAS or ICP) are time consuming.

The cost analysis of DTZ staining protocol showed that the chemical cost per sample is about US$ 0.75 (when 10 g of DTZ approximately costs US$ 25 and 1000 ml of pure methanol approximately costs US$ 12). The cost of the technicians per sample would add another US$ 0.1 (assuming each technician's salary is approximately US$ 225 per month), hence taking the total cost to US$ 0.85 per sample. Thus, this protocol is not only simple and rapid but is also inexpensive when compared to conventional laboratory methods of analyzing grain Zn content. This protocol can, therefore, be effectively used as the method of initial screening, and the genotypes identified for high grain Zn could be subjected to a normal laboratory analysis using AAS or ICP. This would save the cost involved in quantitative estimation of grain Zn. In addition, a highly significant positive correlation ($r = 0.84$; $P < 0.01$) between grain Zn and Fe has been observed in pearl millet (Velu et al. 2007), implying that this method would also be useful for indirect selection of genotypes with high grain Fe. Special care, however, should be taken while using samples with different grain color (grayish colored grains and brownish colored grains), in order to minimize possible interference of the stain color by the background color of the sample.

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