A Rapid Screening Method for Grain Iron Content in Pearl Millet

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Introduction

Enhancing grain iron (Fe) content is one of the effective ways of increasing the Fe intake and reducing the incidence of Fe-deficiency anaemia (Welch and Graham 2002). Large genetic variability for grain Fe content has been reported in many crops (Graham et al. 1999). Pearl millet [*Pennisetum glaucum* (L.) R.Br.] is a major source of dietary energy for millions of people living in the arid and semi-arid tropical regions of Africa and Asia. It has on an average 50 mg kg^{-1} of grain Fe, which is more than wheat, rice and maize. Studies with limited germplasm have shown large genetic variability for this trait, indicating good opportunities to select and/or breed millet genotypes with still higher grain Fe (Jambunathan and Subramanian 1988; Abdalla et al. 1998). The bottleneck in this process is the high cost of Fe estimation. At present Fe estimation is done with digests using Atomic Absorption Spectrophotometry (AAS) and Inductively Coupled Plasma-Atomic Absorption Spectrophotometry (ICP), which require relatively expensive instruments and are time-consuming. These chemical analyses would be prohibitively costly for evaluating a large number of progenies during the course of a breeding program. A procedure based on Perls' Prussian blue stain was proposed for rapid screening of grain Fe content in rice (Prom-u-thai et al. 2003; Krishnan et al. 2003) which involves scoring color intensity in the embryo of cut and treated seeds (with 2% Prussian blue) through a stereo-microscope. The objective of this research was to simplify the method and assess its effectiveness in screening for grain Fe content in pearl millet.

Materials and Methods

Experimental materials. During the dry season of 2004, 120 entries were grown at the International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru, India for estimation of grain Fe content by the AAS method (Jorhem 1993) at the National Institute of Nutrition (NIN), Hyderabad, India. Out of these, 12 pearl millet lines (6 B-lines and 6 partial inbreds) were chosen with a wide range of grain Fe content (30.8–74.7 mg kg^{-1}) and were classified based on the Fe content as high (51.7–74.7 mg kg^{-1}), medium (40.3–40.8 mg kg^{-1}) and low (30.8–35.7 mg kg^{-1}) to standardize the Perls' Prussian blue procedure. Subsequently, the procedure was validated using 20 B-lines (counterparts of the designated A-lines developed at ICRISAT-Patancheru) of pearl millet with a wide range of grain Fe content (32.4–69.0 mg kg^{-1}). The grain samples obtained from sib mating during the dry season of 2004 at ICRISAT-Patancheru, were used for the experiments. Precautions were taken to avoid external contamination of grain with dust in the field and during threshing and cleaning.

Reagents. In rice, the Perls' Prussian blue method with Prussian blue solution of 2% concentration was used in identifying high grain Fe genotypes (Prom-u-thai et al. 2003). The same concentration was initially used in our method and the reagents were prepared as follows.

1) Aqueous potassium ferrocyanide (2%): 10.0 g potassium ferrocyanide was mixed with distilled water, and the volume was made to 500 mL. The solution was transferred into an acid-cleaned brown bottle for storage, which remains stable for 6 months.
2) Aqueous hydrochloric acid (2%): 10.0 mL concentrated hydrochloric acid (HCl) was mixed with distilled water to make the volume 500 mL. This dilute HCl was transferred into a brown bottle for storage, which remains stable for 6 months.
3) Prussian blue solution: This solution was prepared by mixing equal volumes of 2% HCl and 2% ferrocyanide solutions. This solution was freshly prepared every time during testing. The unused solution was discarded.

In the Perls' Prussian blue protocols described for different histopathological reactions, solutions with 1% to 5% of equal volumes of HCl and potassium ferrocyanide solution have been used. Hence, we too tried 1%, 2%, 3% and 5% Prussian blue solutions for staining the lines with high, medium and low grain Fe content in one experiment.

Staining procedure. Dry pearl millet grain samples were ground into flour with a pestle and mortar and 0.5 g of the flour of each sample was transferred into one quarter of a partitioned petri dish. The pestle and mortar were cleaned adequately with distilled water after grinding each sample to avoid contamination with the flour from the previous sample. The Prussian blue solution (10 ml) was poured

onto the flour in each quarter of the petri dishes. The color development was recorded after 10 minutes and the color intensity was visually scored on a 1–4 scale, where 1 = no color; 2 = less intense blue color; 3 = medium blue color and 4 = more intense blue color. The blue color intensity in the flour was compared with the Fe density of genotypes measured through AAS at NIN, Hyderabad, India. Rank correlation between color score and measured Fe density ranks was estimated (Gomez and Gomez 1984).

Results and Discussion

Pearl millet grains flour when treated with 2% Prussian blue solution in the petri dishes produced varying intensity of blue color in genotypes having medium to high Fe content (Fig. 1). In genotypes having a high Fe content (51.7–74.7 mg kg^{-1}), the blue color was more intense than in those having medium Fe content (40.3–40.8 mg kg^{-1}) (Table 1). No color developed in genotypes with low Fe content (31.2–40.6 mg kg^{-1}). These results suggest that Prussian blue staining was effective in differentiating genotypes with high and low Fe content. The rank correlation between measured grain Fe content and the color intensity score was highly significant and positive (r = 0.92; P<0.01), indicating that higher the Fe content in the grain, the more the intensity of blue.

The feasibility of fine-tuning the protocol was tested by varying the concentration (1%, 2%, 3% and 5%) of the Prussian blue solution. But the intensity of blue color did not vary with varying concentrations of Prussian blue solution (Fig. 2) in pearl millet lines with high, medium and low Fe content. However, color development was slow when treated with 1% solution, suggesting that Prussian blue of 2% concentration would be optimum for effective discrimination of pearl millet lines for grain Fe content.

The standardized protocol using 2% Prussian blue solution was validated using 20 diverse B-lines with a wide range of Fe content (32.4–69.0 mg kg^{-1}) (Table 2). Color development was more intense in B-lines with Fe content ranging from 48.9 mg kg^{-1} to 69.0 mg kg^{-1}. On the contrary, no color developed in B-lines having an Fe content from 32.4 mg kg^{-1} to 39.1 mg kg^{-1} with the exception of ICMB 93333, which in spite of having low Fe content (37.3 mg kg^{-1}), showed less intense blue color as against the expected no color. Medium color intensity was noticed in B-lines having an Fe content of 42.9–46.0 mg kg^{-1}. The seed parent ICMB 94555, in spite of having moderate levels of Fe (47.5 mg kg^{-1}), showed less intense blue staining, which could be considered as an exception. All the B-lines having Fe content above the average of 43.4 mg kg^{-1} showed either medium or high intensity blue color staining, indicating that lines with high grain Fe content cannot be missed by this method. There was highly significant and positive correlation (r = 0.91; P <0.01) between the measured Fe content and the color intensity score, again indicating that higher content of grain Fe in the genotypes is associated with intense blue color development.

In general, the intensity of blue color served as a reliable qualitative selection criterion for grain Fe in pearl millet. The method was efficient in separating genotypes

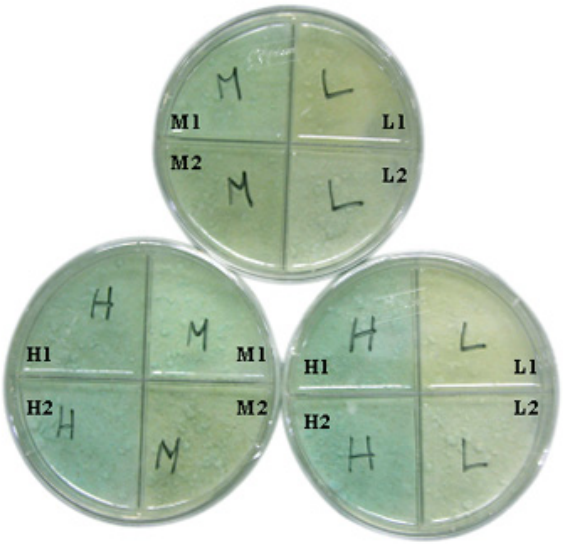

Figure 1. Differential Prussian blue staining of pearl millet grain flour with varying levels (H- high, M- medium and L-low) of grain Fe content. (H1- 863B, H2 - AIMP 92901 S1-15-1-2-B; M1 - MC 94 C2-S1-46-1-1-B; M2 - ICMV 93074 S1-9-1-1-1-B; L1 - 81B; and L2 - ICMB 90111).

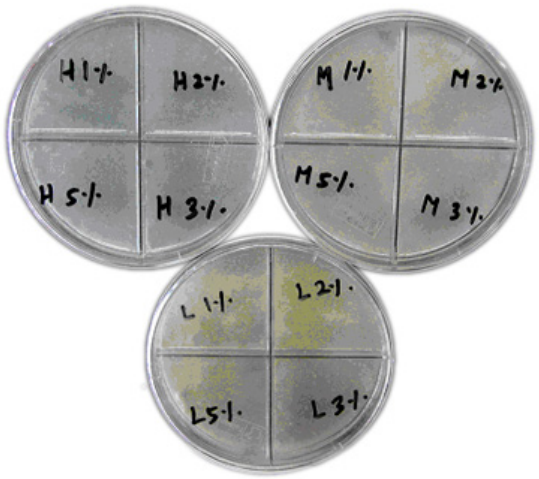

Figure 2. The intensity of blue colour in high (H), medium (M) and low (L) Fe content genotypes with varying concentrations of Prussian blue staining solution.

with high grain Fe from those with low grain Fe. When a large number of germplasm accessions or progenies or breeding lines have to be screened for Fe content, this method will be highly efficient in discarding accessions or progenies with low Fe content or vice versa.

The Prussian blue method could be used at room temperature, and no costly and specific equipment is necessary other than a pestle and mortar or cyclone mill (grinder) and simple glassware. Using a pestle and mortar for grinding, approximately 60–80 samples per day could be analyzed with two technicians; using a cyclone mill, approximately 120–140 samples could be analyzed. Estimation of grain Fe content with AAS or ICP involves higher cost, which ranges from US$ 1 to US$ 10 in various laboratories (excluding the shipping cost of the material).

These chemical procedures are also time-consuming in terms of receiving the results of analyses. The chemical cost per sample in the Perls' Prussian blue protocol used in our study is about US$ 0.4 (when 500 g of potassium ferrocyanide approximately costs US$ 10 and 500 mL

Table 1. Prussian blue staining pattern and grain iron (Fe) content in pearl millet, ICRISAT-Patancheru, Andhra Pradesh, India, dry season, 2004.

Entry	Class	Fe content (mg kg^{-1})	Color class	Color score
ICMS 8511 S1-17-2-1-1-B	Low	30.8	No color	1
ICMV 91059 S1-14-2-4-2-2-B	Low	31.2	No color	1
81 B	Low	32.4	No color	1
ICMB 90111	Low	34.2	No color	1
ICMB 95222	Low	35.2	No color	1
ICMV 91059 S1-58-2-2-2-B	Medium	40.6	Less intense blue color	2
ICMV 93074 S1-9-1-1-1-B	Medium	40.3	Medium blue color	3
MC 94 C2-S1-46-1-1-B	Medium	40.8	Medium blue color	3
ICMB 94111	High	51.7	More intense blue color	4
ICMB 00888	High	56.7	More intense blue color	4
863 B	High	69.0	More intense blue color	4
AIMP 92901 S1-15-1-2-B	High	74.7	More intense blue color	4

Table 2. Prussian blue staining reaction and grain iron (Fe) content in 20 pearl millet B-lines, ICRISAT-Patancheru, Andhra Pradesh, India, dry season, 2004.

B-line	Fe content[1] (mg kg^{-1})	Color class	Color score
81 B	32.4	No color	1
ICMB 90111	34.2	No color	1
ICMB 97333	34.9	No color	1
ICMB 95222	35.2	No color	1
ICMB 98777	35.9	No color	1
ICMB 89111	36.5	No color	1
ICMB 93333	37.3	Less intense blue color	2
ICMB 97111	38.2	No color	1
ICMB 01888	39.1	No color	1
ICMB 01555	42.1	Less intense blue color	2
ICMB 91444	42.9	Medium blue color	3
ICMB 91777	43.7	Medium blue color	3
ICMB 00999	44.3	Medium blue color	3
843 B	46.0	Medium blue color	3
ICMB 94555	47.5	Less intense blue color	2
ICMB 88004	48.9	More intense blue color	4
ICMB 98222	51.3	More intense blue color	4
ICMB 94111	51.7	More intense blue color	4
ICMB 00888	56.7	More intense blue color	4
863 B	69.0	More intense blue color	4

1. Mean = 43.4 mg kg^{-1}; LSD (P = 0.05); CV (%) = 12.3.

concentrated HCl approximately costs US$ 11). 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$ 0.5 per sample. Thus, this is a simple, rapid and inexpensive method compared to both chemical analyses and the method followed in rice. This could be effectively used as an initial method of screening, and genotypes identified for high grain Fe from this protocol could be subjected to actual laboratory analysis using AAS or ICP. This would save the cost involved in quantitative estimation of grain Fe. The highly significant positive correlation ($r = 0.84$; $P < 0.01$) between grain Fe and Zn observed in pearl millet (G. Velu, unpublished) implies that this method would also be useful for indirect selection of genotypes with high grain Zn. However, care should be taken while handling potassium ferrocyanide, which is low toxic under normal conditions, and releases highly toxic cyanide gas when heated.

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