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Dithizone Screening Protocol to Select Promising Rice with High Grain Zinc Content

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Abstract

Estimation or screening of grain zinc (Zn) content is essential for Zn biofortification studies. However, the required sophisticated equipment and human resources are available only in some laboratories of India. Therefore, this study aimed to standardize the conditions to use Dithizone (DTZ) stain to select high grain Zn rice through naked eye for the benefit of the researchers and stakeholders interested to estimate Zn content in rice grain. Various concentrations of DTZ (1 mg/ml to 0.17 mg/ml methanol) were tried in various volumes (25 µl to 100 µl) on intact polished grain, longitudinal sections and varying quantities (25 mg to 100 mg) of polished rice powders. Clear differentiation in intensity of the stain was not observed for both intact polished grain and its longitudinal sections. The combination of 100 mg polished rice powders along with 100 µl of DTZ stain (0.34 mg/ml) was found useful to categorize rice samples as above or below threshold level (24 mg/kg) of grain Zn content with 70 % accuracy in 15 minutes incubation time. The study reports a rapid, simple and inexpensive staining method for rapid screening of rice germplasm for grain Zn.

Keywords: Rice, DTZ, zn staining, grain zinc content, rapid protocol

Introduction

Zinc (Zn) deficiency poses a major challenge to global food and nutritional security, influencing the health and development of vulnerable populations worldwide. Hence, to tackle malnutrition and ensure food security across the world, biofortification strategy was adopted by rice researchers across the globe (https://icar-iirr.org/CRP/). By the end of 2023, more than 443 varieties of biofortified crops were released in more than 40 countries, and over 20.7 million farming households were cultivating these varieties on their farms (Harvest plus Annual report, 2023 www.harvestplus.org). Two decades of consistent research on biofortification by various groups have led to the release of improved zinc-biofortified rice varieties through All India Coordinated Research Project on Rice (AICRPR) biofortification (Sanjeeva Rao *et al.,* 2014; 2020; Anusha *et al*., 2021; Uttam *et al*., 2022; Senguttuvel *et al*., 2023; Sundaram *et al*., 2023; Jaldhani *et al*., 2025).

Dithizone or Diphenyl thiocarbazone (commonly abbreviated as DTZ) ($C_{13}H_{12}N_4S$) is a sulfur containing organic compound (Fischer, 1878). It can form complex with metal ions (**Figure 1**) making it an indispensable reagent for the detection of first, second and third row metal ions of Mendeleev's periodic table, including elements ranging from manganese (Mn) to zinc (Zn); palladium (Pd) to tin (Sn); and platinum (Pt) to bismuth (Bi). Originating from the pioneering work of Fischer *et al*., (1925) and histochemical technique for demonstration of Zn (Okamoto 1942), DTZ was widely utilized due to its ability to provide rapid and accurate determinations of metal ion concentrations. DTZ is used in the localization of Zn in different species *viz.,* algae (Pawlik-Skowronska, 2003), yeast (Bilinski and Miller, 1983), salmon (Paulsen *et al*., 2001), maize (Shobhana *et al*., 2013), pearl millet (Velu *et al.,* 2008) and wheat (Ozturk *et al*., 2006).

As the development of high Zn rice varieties became a part of global sustainable development goals as well as priority of the country, several rice researchers in the country initiated the research programs on Zn biofortification. However, the estimation of Zn in rice grain needs expensive equipment like Atomic absorption spectroscopy (AAS), Inductively coupled plasma (ICP) methods or Energy-dispersive X-ray fluorescence spectrometry (ED-XRF). The non-availability of these equipment is also acting as one of the bottle necks in identifying the promising lines with high grain Zn at many research stations of the AICRPR system. Therefore, the objective of this study is to develop a protocol using DTZ stain to identify high Zn rice lines in zinc biofortification studies.

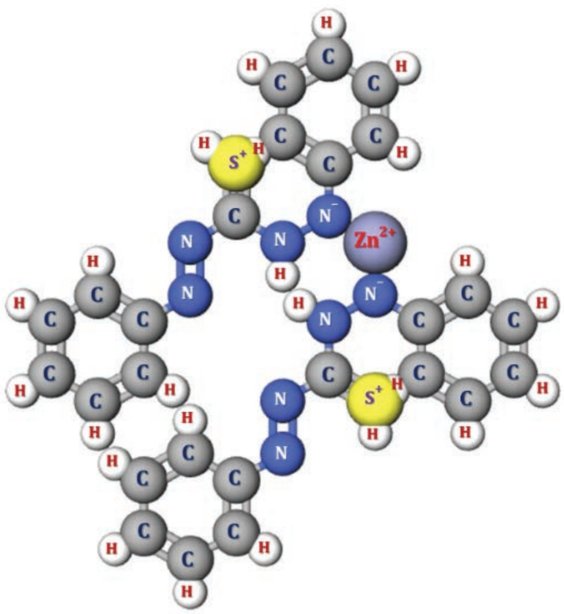

Figure 1: Zinc Dithizonate complex (Obtained from molview.org)

Materials and Methods

Preparation of reagents

DTZ stain (0.5 mg/ml) was prepared using Dithizone (Sigma-43820) dissolved in Methanol (Sigma-Aldrich 34860). Based on the results with this the solution with (0.5 mg/ml) concentration, two more concentrations (0.34 mg/ml and 0.17 mg/ml) of DTZ in methanol were prepared. This stain has a characteristic green color. The solution is unstable when exposed to sunlight, which causes the dithizone to quickly oxidize turning (**Figure 2**) and the solution turn into yellow (Patent Number: WO 2006/116816 A1). Hence, it is recommended to store the solution in an amber colored screw capped glass bottles and this solution can be used for a maximum of 2 weeks, if the initial color is retained.

Figure 2: Freshly prepared DTZ solution (left) and oxidized DTZ solution (right)

Paddy processing and staining with DTZ

The polished rice grain samples were collected with known/predetermined concentration Zn (ppm or mg/kg) ranging from 10 to 44 ppm estimated by ED-XRF. The processing of paddy samples to brown rice followed by polished rice was done with the non-zinc, non-ferrous dehullers and polishers (Sanjeeva Rao *et al*., 2014). Longitudinal sections of polished rice grains were prepared by holding the grain with forceps and cutting with a surgical knife. Approximately one gram of polished rice of each sample was ground manually to fine powder using mortar and pestle. The powders were dried in an incubator or hot air oven maintained at 50 °C for 72 hours to remove the moisture. Various weights of (25, 50, 75 and 100 mg) of samples were taken in a transparent Eppendorf tubes (2 ml capacity) and various volumes (25 to 100 µl) of DTZ solutions (1 mg/ml, 0.5 mg/ml, 0.34 mg/ml and 0.17 mg/ml) that varied in concentration of DTZ stain were added. The contents were mixed thoroughly and kept for incubation at room temperature. The change in color as observed by naked eye and the time required for the change in color were noted for each mixture separately.

Results and Discussion

Considering the importance of high grain Zn in rice biofortification studies, many researchers are breeding to develop high Zn rice lines. However, as mentioned in the introduction, sophisticated equipment are being used often for Zn estimation and they are only available at a few laboratories. The unavailability of reliable Zn estimation facility discourages many rice researchers in the developing breeding material for high grain zinc. This gap needs to be addressed by developing a simple and economical screening method.

DTZ stain appears as a promising approach and it was used in 0.5 mg/ml for wheat (Ozturk *et al*., 2006) and rice (Jaksomsak *et al*., 2015; Duarte *et al*., 2016). DTZ concentration of 0.3 mg/ml was used in maize (Shobhana *et al*., 2013) and peal millet (Velu *et al*., 2008). However, the concentration of DTZ stain varied among these crops which can be due to the range of Zn content in these crops. The DTZ concentration used in wheat which can be similar to rice grain was tried initially in this study as maize and pearl millet grain contains higher amount of Zn.

The visual detection of Zn using DTZ (0.5 mg/ml) was evaluated with varying quantities of rice powder and zinc concentrations to identify minimum quantity of rice powder as well as minimum volume of the stain (**Table 1**). Clear colour variation was not observed in all the four rice samples varying in Zn content from 12 to 42 ppm, while using 25 mg rice powder along with 25 or 50 or 75 µl of DTZ. Similarly, clear colour variation was also not observed while adding when 50 µL of DTZ stain was added to 50 mg rice powder. This indicates that these two sample quantities (25 mg and 50 mg) were insufficient for effective detection. Further, more volume of stain also did not produce the desired results. A modest improvement was observed with 75 mg of powder along with 50 µl DTZ, though the results remained unclear with increase in Zn content. Visual difference between 12 and 22 mg/kg zinc concentrations was observed (**Figure 3**) while using 100 mg of rice powder with 100 µl DTZ (0.5 mg/ml). These findings suggest that rice grain powder sample quantity and DTZ volume as well as concentration are critical for reliable detection of Zn using DTZ in rice grain powder.

Table 1: Effect of DTZ (0.5 mg/ml) concentration with varying Zn content in polished rice grain powders

Weight of rice powder (mg)	Vol of DTZ (0.5 mg/ml) in µl	Known Zn (mg/kg)	Result
25	50	12	No difference between the samples
		22	
		32	
		42	
25	75	12	No difference between the samples
		22	
		32	
		42	
25	25	12	The contents are less to detect
		22	
		32	
		42	
50	50	12	The contents are less to detect
		22	
		32	
		42	
75	50	12	Better than 50 mg powder; but not clear
		22	
		32	
		42	
100	100	12	Colour difference between 12 and 22 mg/Kg was good.
		22	
		32	
		42	

Subsequently, two more concentrations of DTZ (0.17 mg/ml and 1 mg/ml) were evaluated using rice powder samples with known Zn levels ranging from 12 to 44 mg/kg (**Table 2**). With 100 µl of DTZ (0.17 mg/ml), a slight increase in colour intensity was observed between 12 and 22 mg/kg Zn (**Figures 4** and **5**). However, the colour gradation across the full concentration range (12–42 mg/kg) was not distinctly perceptible, suggesting limited sensitivity at this dilution. In contrast, when DTZ was used at a higher concentration (0.34 mg/ml), a more convincing and distinguishable colour variation was observed (**Figure 6**) in 15 min of incubation. Specifically, visible differences in colour were observed between: 12 and 22 mg/kg Zn; 13 and 23 mg/kg Zn; 11 and 21 mg/kg Zn. However, the colour intensity differences between subsequent Zn concentrations (22 and 32 mg/kg; 21 and 31 mg/kg) were less pronounced. Therefore, a set of rice powder samples (100 mg each) varying in Zn content were tested by adding 100 µl of DTZ (0.34 mg/ml) (**Table 3**). Of the 26 samples, the prediction with DTZ stain was matching with known Zn content for 18 samples (70 % accuracy). The final colour developed in the samples was compared with the final colour of reference or check samples that contains target or threshold value of grain Zn content (around 24 mg/kg) in AICRPR biofortification trial (Sanjeeva Rao *et al*., 2014 and 2020).

Table 2: Effect of DTZ (0.17 mg/ml; 0.34 mg/ml) concentration with varying Zn content in polished rice powders

Weight of rice powder (mg)	Vol of DTZ in µl	Known Zn (mg/kg)	Result
100	100 (**0.17 mg/ml**)	12	Colour intensity increased slightly from 12 to 22 ppm; But the colour variation is not fully convincing.
		22	
		32	
		42	
100	100 (**0.34 mg/ ml**)	12	Convincing variation in colour was noticed between 12 and 22 ppm only.
		22	
		32	
		42	
100	100 (**0.34 mg/ ml**)	13	Convincing variation in colour was noticed between 13 and 23 ppm only.
		23	
		33	
		42	
100	100 (**0.34 mg/ ml**)	11	Convincing variation in colour was noticed between 11 and 21 ppm only.
		21	
		31	
100	100 (**1.0 mg/ ml**)	14	Clear colour difference was not noticed.
		24	
		34	
		44	

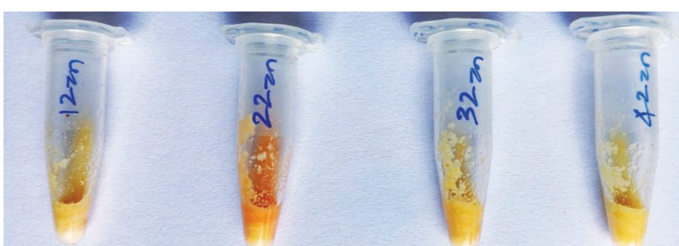

Figure 3: Effect of DTZ (0.5 mg/ml) stain on rice grain powders varying in Zn content

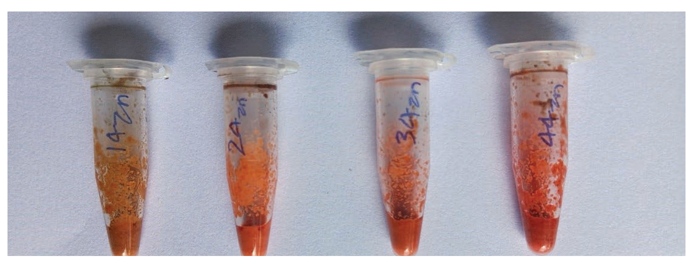

Figure 5: Effect of DTZ (1 mg/ml) stain on rice grain powders varying in Zn content

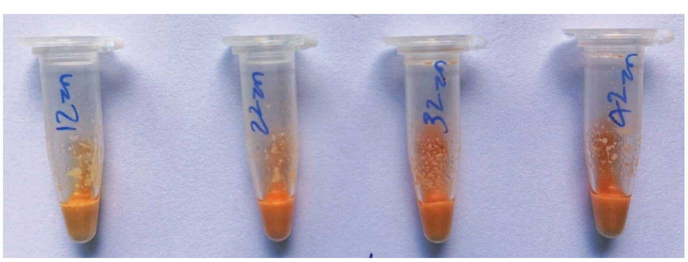

Figure 4: Effect of DTZ (0.17 mg/ml) stain on rice grain powders varying in Zn content

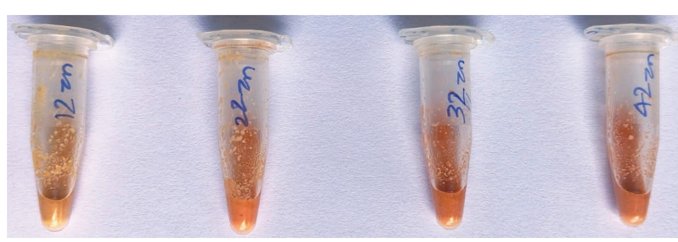

Figure 6: Effect of DTZ (0.34 mg/ml) stain on rice grain powders varying in Zn content

Table 3: Prediction of Zn content in rice using DTZ stain

Sl. No.	Entry	Known Zn concentration (mg/kg)	Prediction with DTZ*
1.	KKR-608	13.9	Below 24 mg/kg
2.	KKR-622	12.5	Below 24 mg/kg
3.	IR-64	17.4	Below 24 mg/kg
4.	RB24/23-160-4-1	28.2	Above 24 mg/kg
5.	KKR-623	11.7	Below 24 mg/kg
6.	RB24/23-126-2-1	36.5	Below 24 mg/kg
7.	KKR-656	13.2	Below 24 mg/kg
8.	RB24/23-23-15-1-1	29.6	Above 24 mg/kg
9.	KKP-669	26.5	Above 24 mg/kg
10.	KARUPPUNEL	35.4	Above 24 mg/kg
11.	RNR-15048	14.1	Below 24 mg/kg
12.	KKR-673	32.7	Above 24 mg/kg
13.	MTU-1010	14.6	Below 24 mg/kg
14.	KKR-670	37.1	Below 24 mg/kg
15.	JAMEER	44.4	Below 24 mg/kg
16.	KKR-671	37.7	Below 24 mg/kg
17.	LR-379	26	Below 24 mg/kg
18.	DODDIGA	26	Below 24 mg/kg
19.	RB24/23-9-1	35.5	Below 24 mg/kg
20.	KALIBORA	42.2	Below 24 mg/kg
21.	KKR-602	17.2	Below 24 mg/kg
22.	KKR-641	18.5	Below 24 mg/kg
23.	KKR-672	23.7	Above 24 mg/kg
24.	KKR-601	16.6	Below 24 mg/kg
25.	KKR-668	28.3	Above 24 mg/kg
26.	KKR-603	12.8	Below 24 mg/kg

*24 mg/kg is the threshold Zn value of AICRP Biofortification trial; Text in bold indicates the prediction with DTZ is correct

In addition to rice powders, DTZ staining was also performed on intact brown and polished rice grains. However, the results were not satisfactory, as discernible color variations were not observed between samples with contrasting, predetermined Zn levels (17 and 34 mg/kg) when assessed visually (**Figure 7**). Subsequently, based on literature as presented in the subsequent paragraphs, longitudinal sections of polished rice grains were prepared and stained with DTZ to localize and distinguish Zn accumulating regions. While the dye successfully stained Zn rich regions, the visual differentiation between samples with varying Zn content remained indistinct and inconclusive. Given these limitations, this study recommends to use polished rice powders over intact/longitudinal sections of rice grains.

In rice crop, in contrast, clear differentiation in the stained areas was reported in the longitudinal sections of Nam Roo (31 mg/Kg) and RD21 (19 mg/Kg) in freshly prepared DTZ (0.5 mg/ml methanol) solution and the variation in the stained area was converted to DTZ index, which was used to predict the Zn content in the samples with 79 % accuracy (Jaksomsak *et al.,* 2015). Similarly, embryo regions were stained more intensely with DTZ (0.5 mg/ml methanol) followed by aleurone layer and endosperm (Duarte *et al*., 2016). Accordingly, they converted the stained regions separately into staining intensity index using image analysis software and predicted the zinc content of rice grains with 70 % accuracy in the rice samples.

In wheat crop, higher accumulation of Zn in embryo was also observed in grain with DTZ (0.5 mg/ml) followed by aleurone layer and endosperm (Ozturk *et al*., 2006). Further, they observed variation in DTZ stain intensity with increase in zinc content wheat grain powders (200 mg) with 200 µl of DTZ stain (0.5 mg/ml).

In maize crop, 5 ml of DTZ (0.3 mg/ml) was added to 1 gram of grain powder and the color intensity of the mixture was visually scored (1 no or little stain to 5 very intense color) after 15 minutes of incubation with an accuracy of 89 % for Zn content (Shobhana *et al*., 2013).

In peal millet crop, 5 ml of DTZ (0.3 mg/ml) was added to 1 gram of grain powder and the color intensity of the mixture was visually scored (1 less intense red color to 3 more intense color) after 15 minutes of incubation (Velu *et al*., 2008).

The demerits of this method are permanent loss of sample due to conversion of the grains to powders and predicts only whether the grain zinc content is above or below the threshold value. The variation in stain intensity in photographs is inferior to the actual intensity observed by the naked eye. The DTZ works well for polished rice powders and further studies are required for brown rice (particularly colored rice).

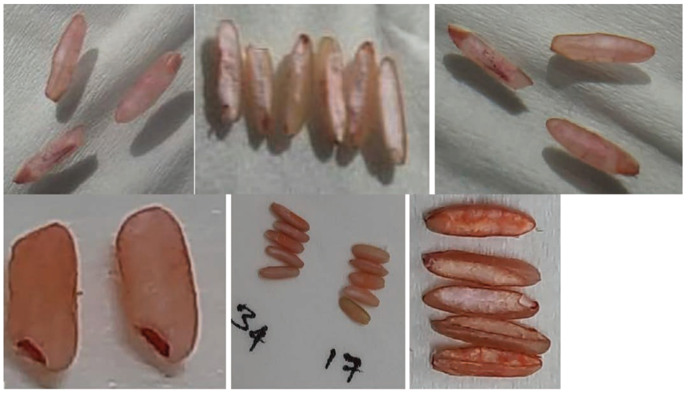

Figure 7: Effect of DTZ (0.34 mg/ml) on intact and longitudinal sections rice grains. 34 and 17 represent grain zinc concentration in mg/kg

Conclusion

The standardized minimum amount (100 mg) of rice powder and minimum volume (100 µl) of 0.34 mg/ml DTZ concentration as identified in this study can be useful to select promising rice samples having with grain Zn content equal to or above 24 mg/kg in rice breeding programmes. The DTZ concentration of 0.34 mg/ml is more suitable for preliminary screening, suggesting its potential utility (70 %) for semi quantitative estimation of Zn in polished rice grain powder. Further, it is also important to compare with check samples with known Zn concentration above and below the threshold value of Zn. Simultaneously, it is worth to mention that the Zn content in rice samples categorized as biofortified can also vary with the location. Moreover, the processing of paddy to polished rice grain powders must be carefully carried out to avoid external contamination of samples with zinc content.

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