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Determination of zinc in rice grains using DTZ staining and ImageJ software

Rômulo F. Duarte [a, b, *], Chanakan Prom-u-thai [a, c, **], Douglas C. Amaral [b], Valdemar Faquin [b], Luiz R.G. Guilherme [b], André R. Reis [d], Eduardo Alves [e]

[a] Agronomy Division, Department of Plant Science and Natural Resources, Faculty of Agriculture, Chiang Mai University, Chiang Mai, 50200, Thailand
[b] Department of Soil Science, Federal University of Lavras, P.O. Box 3037, Lavras, MG 37200-000, Brazil
[c] Lanna Rice Research Center, Chiang Mai University, Chiang Mai, 50200, Thailand
[d] Biosystems Engineering, Faculty of Science and Engineering, Sao Paulo State University, Tupã, SP 17602-496, Brazil
[e] Department of Plant Pathology, Federal University of Lavras, P.O. Box 3037, Lavras, MG 37200-000, Brazil

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ABSTRACT

Dithizone (DTZ) staining is a rapid, simple, and inexpensive method that allows the histochemical localization of Zn. In this study, we evaluated genotypic variation in Zn concentration in brown rice grown in the field using atomic absorption spectrophotometer (AAS) as DTZ staining quantified using ImageJ software. We used dehusked grains from upland rice accessions widely cultivated in Brazil. The DTZ staining showed that the concentration of Zn varied within and between rice grains. The staining intensity index (*Y*) provided differences in localization of Zn across the grain regions. Zn concentration in brown rice varied in multiple regression analysis, showing major differences in index weighted by stained area of each region (*YAW*) for the embryo, endosperm, and aleurone, especially in the endosperm and aleurone regions due to a large portion of the area in the kernel. The total *YAW* among rice accessions was positively correlated with Zn concentration in grains by chemical analysis. The DTZ staining associated with imageJ software is a promising to estimate Zn concentration in different grain tissues. Thus, this method may be useful for rapid screening of rice germplasms.

1. Introduction

Zinc (Zn) is an essential micronutrients required for normal and healthy growth of plants, animals, and humans (Alloway, 2008). Zinc deficiency occurs in about half the world's population, and causes general problems associated with growth and development including birth defects in pregnant women, stunted growth of children, and increased susceptibility to infectious diseases (Cakmak, 2008; Graham et al., 2012; Prasad, 2012). The consumption of agricultural products with inadequate amounts of Zn levels is considered to be the leading primary reason for Zn deficiency in humans, especially in developing countries (Alloway, 2008; Pfeiffer and McClafferty, 2007; White and Broadley, 2009; Sharma et al., 2013). Therefore, increasing Zn concentration in crops such as rice, an important source of energy for more than three billion people living in Asia, could provide a strategy for decreasing the incidence of Zn deficiency in regions where rice is the staple crop (Bouis and Welch, 2010).

Significant variation in grain Zn concentration can be found among different rice genotypes. The concentration of Zn ranged between 15.9 and 58.4 mg kg^{-1} for 939 brown rice samples evaluated at IRRI (Graham et al., 1999) and from 13.32 to 43.65 mg Zn kg^{-1} in 274 samples from China's germplasm (Jiang et al., 2008). Since the target Zn concentration for biofortification strategies is 28 μg g^{-1} dry weight for rice grains it has been suggested that this might be achieved through plant breeding (Bouis and Welch, 2010; White and Broadley, 2011).

Studies have reported variation in Zn concentrations in different tissues. For instance, the highest Zn concentration (179 mg kg^{-1})

* Corresponding author. Department of Soil Science, Federal University of Lavras, P.O. Box 3037, Lavras, MG 37200-000, Brazil.
** Corresponding author. Department of Plant Science and Natural Resources, Faculty of Agriculture, Chiang Mai University, Chiang Mai, 50200, Thailand.
E-mail addresses: rfduarte83@gmail.com (R.F. Duarte), chanakan15@hotmail.com (C. Prom-u-thai).

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was found in the embryo, followed by the aleurone (51 mg kg^{-1}) and the endosperm (21 mg kg^{-1}) (Saenchai et al., 2012). The variation in Zn concentration among different grain tissues affects the concentration in the whole kernel in rice (Hansen et al., 2009; Saenchai et al., 2012) in a manner similar to wheat (Cakmak et al., 2010). Therefore, a technique that takes into account the variation among the tissues and their contribution to whole grain Zn concentration should be useful in germplasm evaluation and breeding. However, milling chemical analysis are high cost per samples and request expensive infrastructure (Pfeiffer and McClafferty, 2007). Staining with dithizone (DTZ) is an inexpensive technique to detect Zn in tissues (Ozturk et al., 2006; Velu et al., 2008; Shobhana et al., 2013). Dithizone is a Zn-chelating agent that binds Zn complex through an intense red color produced by Zn-dithizone complex (Cakmak et al., 2010; Ozturk et al., 2006). Dithizone stains has also been used to localize Zn grain (Jaksomsak et al., 2014; Prom-u-thai et al., 2010). However, these previous studies did not quantify the content of Zn in each tissue to the total grain Zn concentration. This study aimed to: (i) to estimate the intensity of DTZ staining in brown rice grain tissues using ImageJ software, (ii) to estimate the accuricity of DTZ staining and correlation with total Zn in grains, and (iii) to evaluate genotypic variation in Zn concentration of rice grain using chemical and DTZ staining.

2. Materials and methods

2.1. Plant material and sample preparation

Seeds of six upland rice (*Oryza sativa* L.) accessions (Table 1) were provided by Upland Rice Breeding Program, which is developed in Minas Gerais state (Brazil); EPAMIG (Agriculture and Livestock Research Institute), UFLA (Federal University of Lavras), and EMBRAPA (Brazilian Agricultural Research Corporation). A field experiment was undertaken in 2011/2012 in a paddy field at Value for Cultivation and Use (VCU) in the EPAMIG Experimental Station (EELA), in Lavras, MG, Brazil (latitude: 21°14′ S; longitude: 45°00′ W and elevation: 918.841 above msl) during the rice growing season. VCUs are made for a particular regional scope, or for one or more Brazilian states and have usually been applied in plant research for annual plants to select the best accessions based on genotypic value for cultivar recommendation. Rice plants were grown in sandy clay soil under upland conditions with additional irrigation at EELA. The experimental design was a randomized complete block with three replications. Each plot consisted of five rows of 5.0 m, spaced 0.4 m apart and a density of 80 seeds per metre. Basal fertilization consisted of the mixture of 400 kg ha^{-1} of NPK fertilizer (08-28-16) + 0.5% Zn and top dressing with 100 kg ha^{-1} of N (ammonium sulfate) was applied in two equal portions (25 and 45 days from sowing) (typical fertilization for trials testing the Value for Cultivation and Use – VCU). Rice grains were harvested at full maturity for Zn analyses.

2.2. Chemical analysis

Rice grains from ten plants per plot were harvested and oven-dried at 65 °C until the grain reached a constant weight prior to analysis. Grain was dehusked in a testing husker (model MT, SUZUKI) to obtain brown rice caryopses (unpolished). For Zn analysis, 100 mg samples of brown rice caryopses were digested using 4 mL of concentrated HNO_3 and 2 mL of concentrated $HClO_4$ (Sigma–Aldrich, Saint Louis, MO, USA) at 120 °C for 1 h and then at 220 °C until $HClO_4$ fumes were observed. Total Zn concentrations in the samples were determined by atomic absorption spectrophotometry (AAS, PerkinElmer Inc., San Jose, CA, USA) (Malavolta et al., 1997) with three replications. Tomato leaf (SRM 1573A) and rice flour (SRM 1568A) standards (National Institute of Standards and Technology, Gaithersburg, MD) were digested and analyzed along side the rice samples to ensure accurate and reliable analytical data.

2.3. Diphenylthiocarbazone (dithizone) staining

About twenty rice grains were dehusked manually, excised longitudinally along the crease by a scalpel, and then submerged in freshly-prepared DTZ solution, by dissolving 1,5-diphenyl thiocarbazone (Merck) (500 mg L^{-1}) in methanol (reagent grade) for 30 min, as described previously (Ozturk et al., 2006). Samples were rinsed thoroughly in distilled deionized water and blotted dry using tissue paper.

2.4. Image acquisition and processing

Rice grains stained using DTZ were photographed at a 6× magnification using a stereoscopic zoom microscope (Nikon SMZ 1500, Japan) and a Camera Control Unit (Nikon, SU-1). The 24 bit-depth images were analyzed on a desktop or laptop computer using ImageJ software (Ferreira and Rasband, 2012). The intensity of staining was measured through the RGB color space (Red, Green and Blue) defined by formula staining intensity values expressed as $R + G + B/3$. Intensity data represented the relative density of Zn in the grains, and was scored from 1 (less intense color), 2 (medium intense color), 3 (intense color) to 4 (very intense color) in accordance with the intensity of staining (RGB values, scale from 0 to 255).

2.5. Staining intensity analysis

Staining intensity values ($R + G + B/3$) from RGB images were evaluated by ImageJ software and then scored as 1, 2, 3 or 4, according to frequency distribution in 4-class interval as following in Eq. (1):

Table 1
Zinc concentration in grains and information about 6 upland rice accessions used in our study.

Identification	Zn (mg kg^{-1})[a]	Accessions (release year)[b]	Recommendations[c] (Brazilian states)
1	29.6 ± 0.59	BRS Esmeralda (2012)	MT, GO, MA, MG, PA, PI, RO, RR, TO
2	31.6 ± 1.27	Line MG 1097 6	–
3	32.3 ± 1.07	BRSMG Relâmpago (2007)	MG
4	27.2 ± 0.55	BRSMG Caravera (2007)	MG
5	36.5 ± 1.14	Line CMG 1510	–
6	30.1 ± 0.93	BRSMG Curinga (2004)	MG, GO, MT, MS, TO, AM, RO, MA, PA, PI

[a] Zinc concentration in brown rice (husks removed) were determined by atomic absorption spectrophotometry (AAS).
[b] Accessions included 4 cultivars and 2 lines of upland rice.
[c] Refers to some locations of the regions where the cultivars or lines are tested and then suggested for growing.