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

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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})

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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.

$$\begin{aligned}
&\text{score 1} = [\text{Min}(R+G+B/3) - (R+G+B/3)_1 >;\\
&\text{score 2} = [(R+G+B/3)_1 - (R+G+B/3)_2 >;\\
&\text{score 3} = [(R+G+B/3)_2 - (R+G+B/3)_3 >;\\
&\text{score 4} = [(R+G+B/3)_3 - \text{Max}(R+G+B/3) >;
\end{aligned} \quad (1)$$

Where "1>" mean the superior limit of the first class interval (score 1) as well as "2>" mean the superior limit of the second class interval (score 2), etc. Moreover, Min($R + G + B/3$) and Max($R + G + B/3$) represents the lowest and highest staining intensity values ($R + G + B/3$), respectively, measured by ImageJ software analysis.

The Eq. (2) below was adapted according to the equation used by Pintasen et al. (2007) to calculate the staining intensity index among different seed lots of upland rice germplasm. Therefore, to calculate the staining intensity index (Y) within each region (embryo, endosperm or aleurone) of the rice seeds, Eq. (2) was used:

$$Y = \overbrace{\frac{(\text{Intensity }(\%) \times \text{SEm})}{100}}^{\text{Embryo}} \text{ or } \overbrace{\frac{(\text{Intensity }(\%) \times \text{SEnd})}{100}}^{\text{Endrosperm}} \text{ or } \overbrace{\frac{(\text{Intensity }(\%) \times \text{SAl})}{100}}^{\text{Aleurone}} \quad (2)$$

where:

Y = Average staining intensity within each regions of the rice seeds.

The percentage of the score region (%SEm, %SEnd and %SAl) was set from Eq. (1), where score 1, score 2, score 3 and score 4 were represented by 25%, 50%, 75% and 100%, respectively. The color intensity of each rice seed (SEm; SEnd and SAl) was visually scored on a 1–4 scale as following: 1 (less intense color), 2 (medium intense color), 3 (intensity color) and 4 (very intense color) (see Subsection 2.4).

The index weighted by stained area of each region (*YAW*) was obtained by multiplying the staining intensity index (Y) within each stained region determined by the imageJ software with their area in mm^2, i.e., as following in Eq. (3):

$$\begin{aligned}
YAW &= (Y \text{ region} \times \text{area}) \text{ and Total } YAW\\
&= \sum[(Y \text{ embryo} \times \text{area}) + (Y \text{ aleurone} \times \text{area})\\
&\quad + (Y \text{ endosperm} \times \text{area})].
\end{aligned} \quad (3)$$

2.6. Statistical analysis

Analysis of variance (ANOVA) was carried out among the treatments using SISVAR version 5.3 software and the coefficient of variation was also calculated to estimate the reproducibility of results. Differences between treatments were compared by the LSD test ($p < 0.05$). Rice grain Zn concentration by chemical analysis and the intensity of DTZ staining were subjected to evaluate the linear correlation among all varieties.

3. Results

3.1. Concentration and localization of zinc in rice grains

A genotypic variation in Zn concentration was found in six Brazilian upland rice accessions for brown rice (husks removed), which ranged from 27.2 to 36.5 mg kg^{-1} (Table 1). Considering Zn concentration in brown rice, which is the edible portion after dehusking process, the highest Zn concentration was found in accession 5 (36.5 mg kg^{-1}), whereas accession 4 had the lowest (27.2 mg kg^{-1}). Moreover, the DTZ staining showed comparable intensity of Zn localization when viewing the whole intact kernels, but the staining intensity of each grain region was more clearly differentiated in the longitudinal sections of the kernels, which was chosen for staining intensity analysis of rice grain among different regions and accessions through ImageJ software (Supplementary Fig. 1).

3.2. Staining intensity through image analysis

The images of each stained section were rescored using the staining intensity values ($R + G + B/3$). The ImageJ software was used to analyze the $R + G + B/3$ values from RGB images in different regions of rice seed studied (embryo, endosperm and aleurone zones) (Fig. 1A). There were different levels of staining intensity (RGB intensity × distance) among regions of the seeds such as embryo (Fig. 1B), aleurone layer (Fig. 1C) and endosperm (Fig. 1D), yet they were in concordance with the sensitivity of red tones perceptible to the human eye.

The $R + G + B/3$ values from RGB images were used to calculate the staining intensity index (Y) as shown in Eq. (2). The significant variation of the index was found among seed regions (the embryo, endosperm, and aleurone) of six rice accessions (Fig. 2). Half of the analyzed rice accessions (numbers 1, 3, and 4), the higher index ($p < 0.05$) was found in the embryo compared to the aleurone and endosperm regions. On these three accessions, the index follows the order embryo > aleurone > endosperm. However, the highest index for the endosperm and aleurone regions was found for the accession 5. There was no difference of the index among the 3 regions in accession 6.

The area of each stained region was different within and between accessions (Fig. 3A). The stained area of the endosperm was about 10 times larger than of the embryo and aleurone regions in all accessions. The index weighted by stained area of each region (*YAW*) was used to represent the staining intensity of each region in the whole kernel.

Combining the *YAW* for the 3 regions gave whole grain *YAW* that indicated significant variation in the amount of Zn in the grain among the 6 accessions studied ($p < 0.05$) (Fig. 3B). Accession 5 had the highest whole grain *YAW* and the lowest was found in accessions 1, 2, 4, and 6. The total *YAW* among 6 accession was positively correlated with Zn concentration in brown rice kernel by chemical analysis ($r^2 = 0.74$, $p < 0.05$) (Fig. 4).

4. Discussion

4.1. Concentration and localization of zinc in rice grains

The chemical analysis showed a variation of Zn concentration in the kernel of 6 rice accessions studied, which was reported previously in several germplasms (Graham et al., 1999; Saenchai et al.,

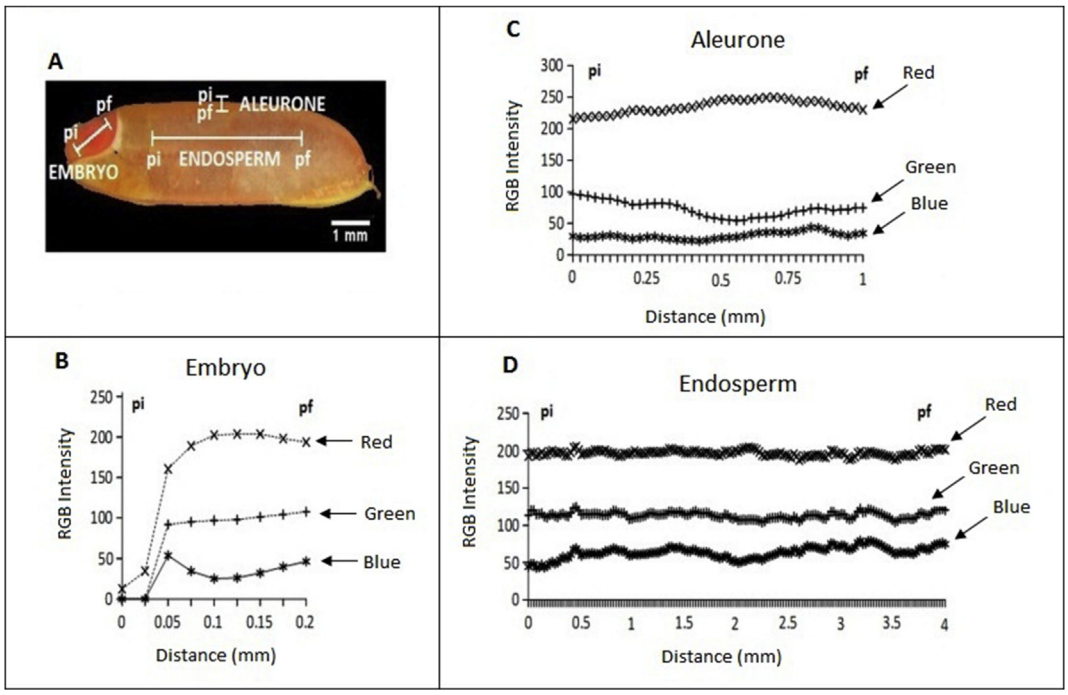

Fig. 1. Stereo-micrographs of longitudinally-cut grains surface stained by dithizone (DTZ). The intensity of staining represents the relative density of Zn in the grains where the longitudinal section of the grain kernel (A) shows the measurement area of the grain. All graphics represented the intensity curves according to different seed regions studied such as embryo (B), aleurone (C) and endosperm (D) by ImageJ software. Distance on the *x*-axis shows the length of the studied grain regions. Points: initial (pi) and final (pf).

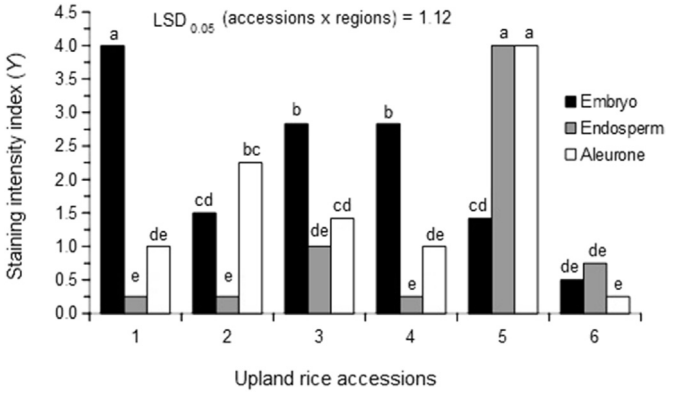

Fig. 2. The staining intensity index (*Y*) among grain regions of 6 upland rice accessions from Brazil. Values shown represent means (bars) calculated from three independent replications.

2012). The variation of Zn in grains among rice accessions probably depends on the ability of Zn to mobilize from one part of the plant to the others such as from the husk to the grain. Recently, Yoneyama et al. (2010) reported that Zn redistribution for the grains and husks might be via the phloem after mobilization from the flag leaf and leaves below the flag leaf. However, several mechanisms may affect Zn remobilization such as the plant's nitrogen nutrition, the micronutrient transporters genes (e.g., ZIP family), the pH of the phloem sap and importantly, the forms of Zn-chelators as nicotianamine (NA) and deoxymugineic acid (DMA) in the plant (Bashir et al., 2012; Cakmak et al., 2010; Clemens et al., 2013). Mediated by nicotianamine synthase (*NAS*) genes, NA plays an important role in the intercellular and long-distance transport of Zn and has also contributed to Zn mobilization into the endosperm (Clemens et al., 2013; Takahashi et al., 2009). However, it has also been indicated that Zn concentration in the grain was affected by the proportion of Zn in different grain regions both in rice (Hansen et al., 2009; Saenchai et al., 2012) and wheat (Cakmak et al., 2010). Thus, the appropriate localization of Zn intensities in different parts of a seed is a key factor for Zn concentration in the whole kernel of rice. In our study, the staining of longitudinal grain section with DTZ indicated that Zn is mostly localized in the embryo and aleurone layer parts, which agrees with previous investigations in several kinds of cereal grains by using the similar staining method (Cakmak et al., 2010; Ozturk et al., 2006; Shobhana et al., 2013; Velu et al., 2008). On the other hand, when the staining intensity is observed in tissues of rice grains by human eyes, it can be difficult to distinguish, especially when dealing the narrow variation among grain tissues. Therefore, the staining intensity carried through DTZ method and being visualized by imageJ software may solve part of these problems aforementioned. In that perspective, we suggest this technique as an important tool for studies that involve the localization of Zn in different regions of rice grains.

4.2. Assessing staining intensity through imageJ analysis

Several approaches have been developed to investigate nutritional features in grains of cereal crops by using the staining technique (Ozturk et al., 2006; Jaksomsak et al., 2014; Prom-u-thai et al., 2010; Velu et al., 2008; Shobhana et al., 2013), which generate images. However, to our knowledge, the use of ImageJ software for specific analysis and measurement of stained grains is still rare. The measurement of staining intensity (*I*) by ImageJ software analysis as demonstrated by profile of intensity was calculated to the staining intensity index (*Y*) for the comparable of the intensity level as investigated in the previous studies (1: less intense; 2: medium intense; 3: intense; 4: very intense) (Jaksomsak et al., 2014; Pintasen et al., 2007). When we used these indices, our results of rice staining showed that on embryo surface of analyzed grains had the highest Zn concentration compared with the endosperm and

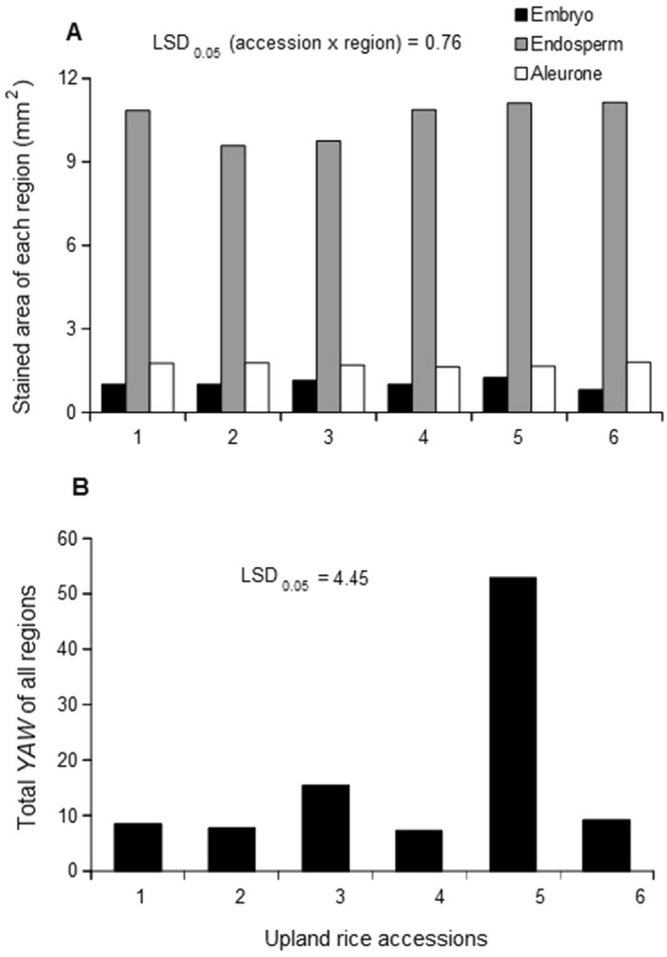

Fig. 3. The stained area of each grain regions (A) and total *YAW* of rice kernel (B) from 6 upland rice accessions. Total *YAW* is the sum of the staining intensity index weighted by stained area of each region (*YAW*) in the embryo, aleurone layer and endosperm as in the following Eq. (3): Total *YAW* = Σ [(*Y* embryo × area) + (*Y* aleurone × area) + (*Y* endosperm × area)]. For stained area, values shown represent means (bars) calculated from three independent replications.

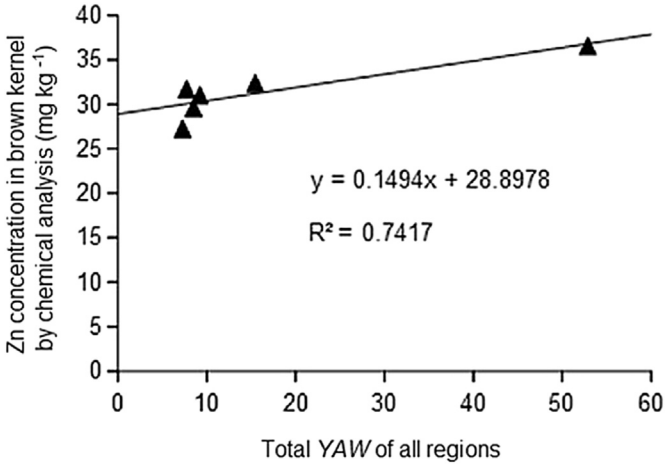

Fig. 4. Relationship between Zn concentration in brown rice from chemical analysis and total *YAW* of all regions from 6 upland rice accessions. Total *YAW* is the sum of the staining intensity index weighted by stained area of each region (*YAW*) in the embryo, aleurone layer and endosperm as in the following equation: Total *YAW* = Σ [(*Y* embryo × area) + (*Y* aleurone × area) + (*Y* endosperm × area)].

aleurone layer in most accessions. This is consistent with recent findings of advanced method, which revealed a similar distribution pattern of Zn in the embryo of rice grains by Synchrotron X-ray fluorescence microscopy (XFM) (Lu et al., 2013; Kyriacou et al., 2014; Takahashi et al., 2009). However, the XFM technique may not be as easily available or accessible compared to the staining method addressed in this essay together with the ImageJ software. Previous studies reported that the distribution of Zn was considerably localized in the embryo and aleurone layer of wheat (Ozturk et al., 2006) and rice kernels (Lu et al., 2013). However, the higher *YAW* index found in the endosperm of the grain compared with the other regions was probably due to the much larger endosperm surface area in those grains (Fig. 3A), demonstrated by the images from our analysis. The higher Y in the aleurone and endosperm than in the embryo of accession 5 contributed the large portion to *YAW* could be due to cross contamination from other regions of embryo and aleurone layer which can be occurred during soaking and sectioning of rice grain before the staining procedures. The contamination of Zn from other region to the endosperm resulted in high staining intensity value R + G + B/3 which was used to calculate Y. This cause of the higher endosperm Y, rather than a larger endosperm surface area, that contributed to a higher *YAW* for accession 5 than others. Therefore, attention should be made when preparing the grain samples for Zn staining to avoid contamination between the regions of rice grain and/or other sources e.g., lazor blade and laboratory glass wares.

4.3. Correlation analysis

The significant variation of Zn concentration in brown rice kernel was complemented by measuring the staining intensity weight by stained area of the embryo, endosperm, and aleurone regions. The staining intensity method showed that various regions of the grain contained differentiated amounts of Zn concentration. The contribution was greater from the endosperm and aleurone regions due to their larger area compared with embryo in the image analysis. The total Zn concentration in the whole caryopsis is contributed by the staining intensity Zn in all regions as it was observed from the significant relationship between total Zn concentration in the caryopsis and the total *YAW* ($r^2 = 0.7417$, $p < 0.05$) (Fig. 4). If the total *YAW* is established from the staining intensity of DTZ, for example total *YAW* = 30, the concentration of Zn in brown rice caryopsis would be estimated as 33.38 mg kg^{-1} which could be valuable and convenient in the conventional laboratory where atomic absorption spectrophotometry is not existed. However, there was a weak point that could be observed in this method. For example, a wide gap between the total *YAW* and grain Zn concentration of both brown rice caryopsis and rough rice of accession 5 was observed and could be explained by the sensitivity of the staining intensity and *YAW* calculations. As described above that *YAW* is the index use to represent the staining intensity of each region in the whole kernel calculated by multiplying the staining intensity index (*Y*) within each stained region determined by the imageJ software with their area. Therefore, the indication of the staining intensity and the stained region is highly sensitive to the total *YAW*. For example, if there is any contamination of Zn staining across the regions and over calculation from the stained regions would make high Y value and consequently resulted in high total *YAW* as appeared in accession 5. Otherwise, our methodology indicates that manipulating the ImageJ software together with the DTZ staining can be efficiently used to assess the variation of Zn concentration between rice accessions and within the grain tissues from seeds, e.g., the endosperm and aleurone layer. It is a rapid, easy and economical method to evaluate Zn concentration in rice grain, especially when dealing with large germplasms (Shobhana

et al., 2013; Velu et al., 2008). This method allows plant breeders to determine the relative concentrations of Zn in rice within and between seed lot when working with rice samples that are genetically diverse (Pintasen et al., 2007; Prom-u-thai et al., 2003). However, thorough and specific evaluations of the small regions such as the embryo or the scutellum would be necessary to improve this method for further research and development.

5. Conclusions

This study confirms that the variation of Zn concentration can be distinguished among rice accessions, seed lots, and grain tissues by using DTZ staining together with the ImageJ software analysis. The total staining intensity multiply by the stained regions (*YAW*) can be used to estimate grain Zn concentration, which can be convenient among the conventional laboratory without Atomic Absorption Spectrophotometer. The staining method is easy, rapid, and economically viable and ImageJ software is freely accessible imaging program. This is an important finding for rice breeders interested in developing high-density Zn genotypes to solve the problem of Zn deficiency among the world's population. The screening procedure may be carried out even in large number of samples without the problem of small amount per sample of rice, a problem that often occurs when performing chemical analysis. Even though, cross-sectioning is still a manual rather than an automated operation which would limit the number of samples that can be analyzed, further research requiring development of the specific tools and further refined by calibrating the staining surface to identify more closely the contribution of different tissues to total grain Zn.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jcs.2015.11.006.

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