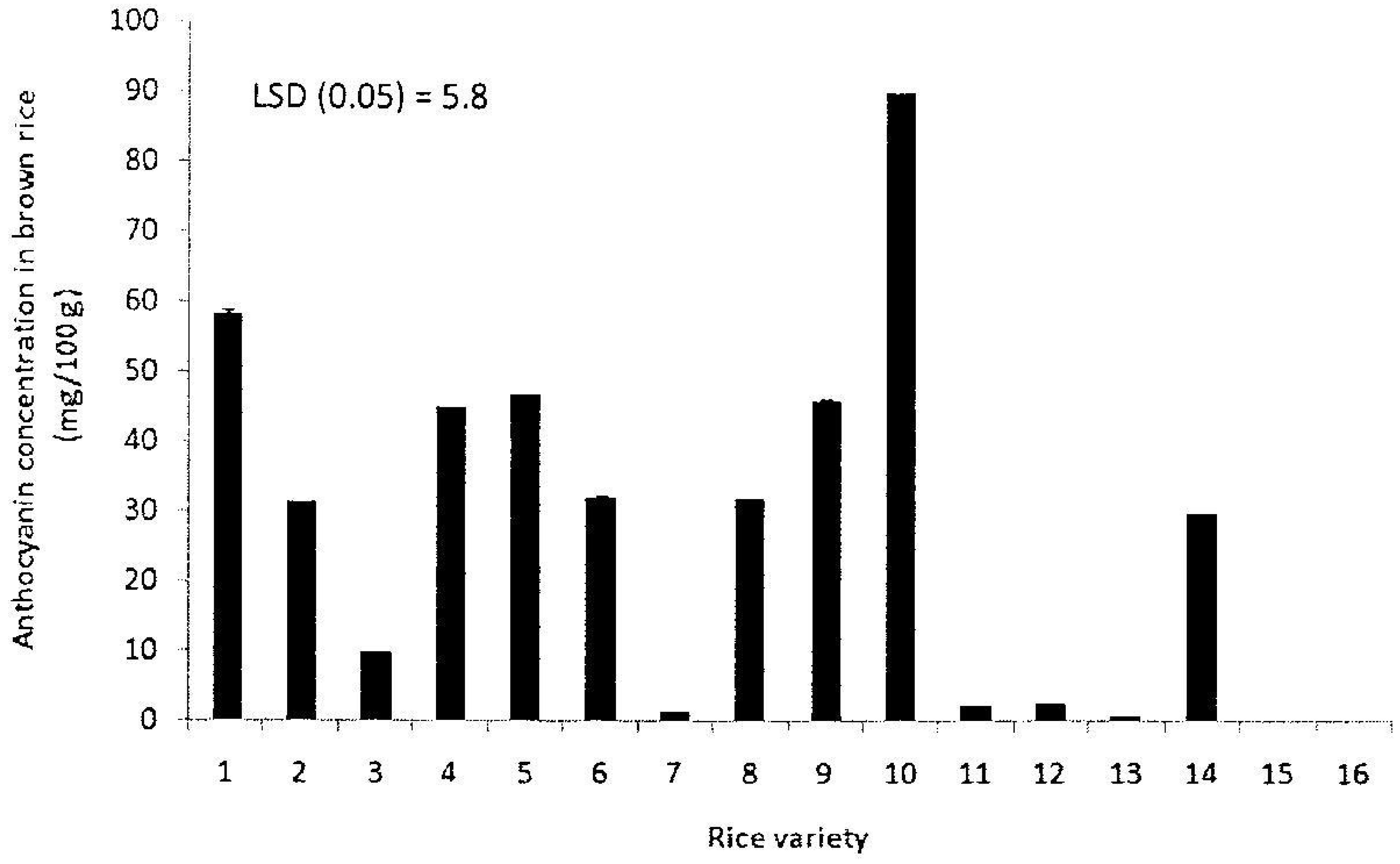
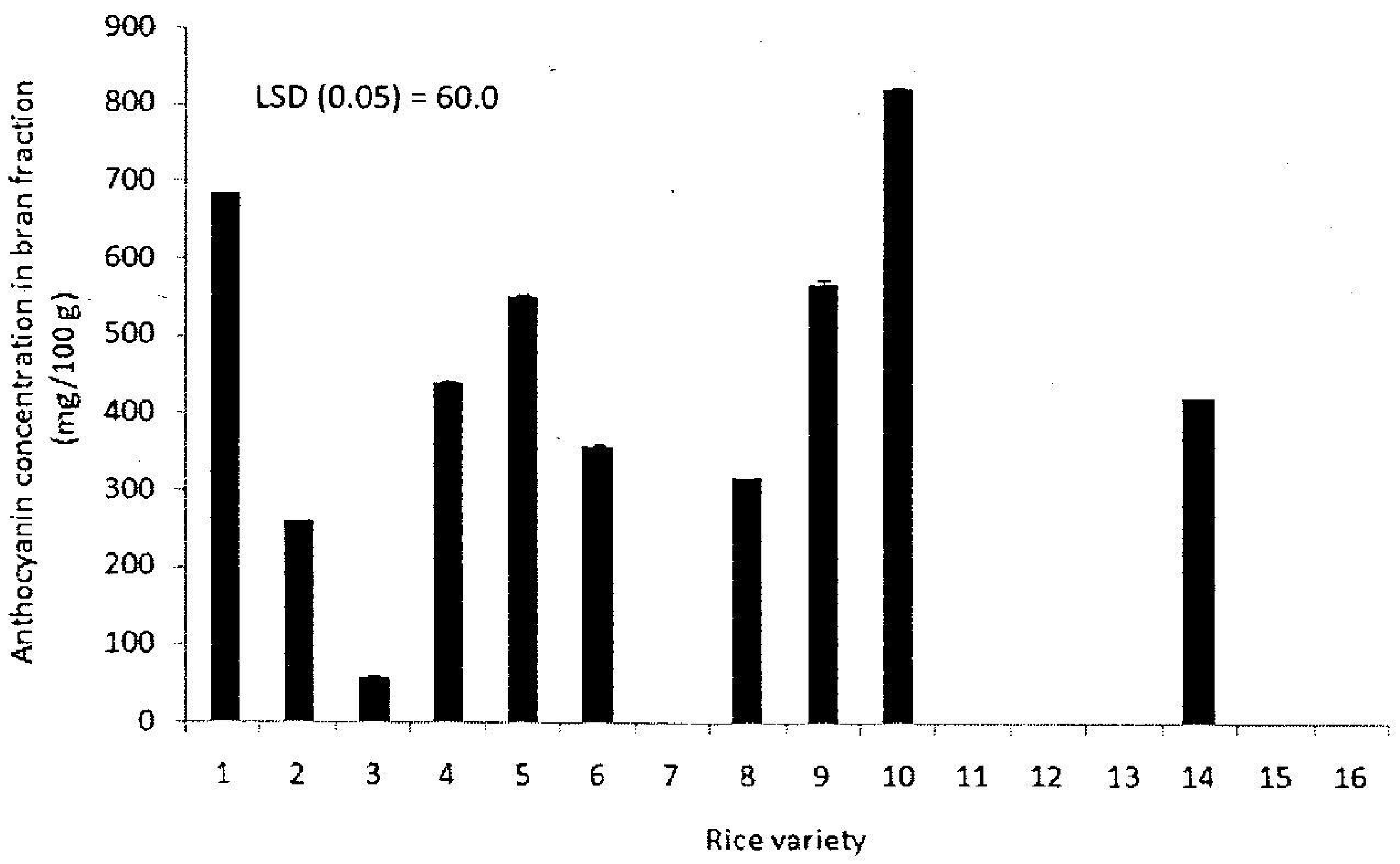
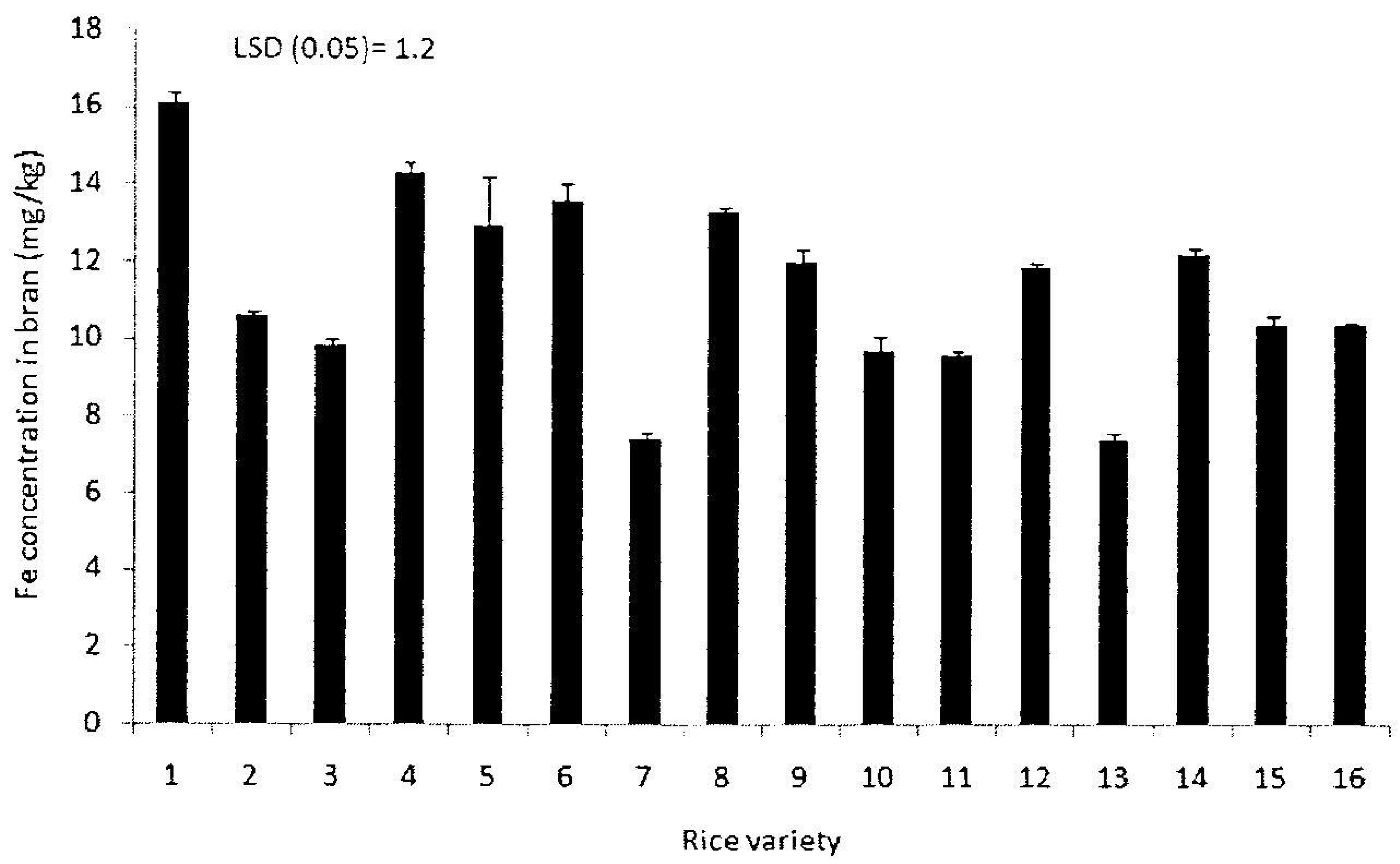


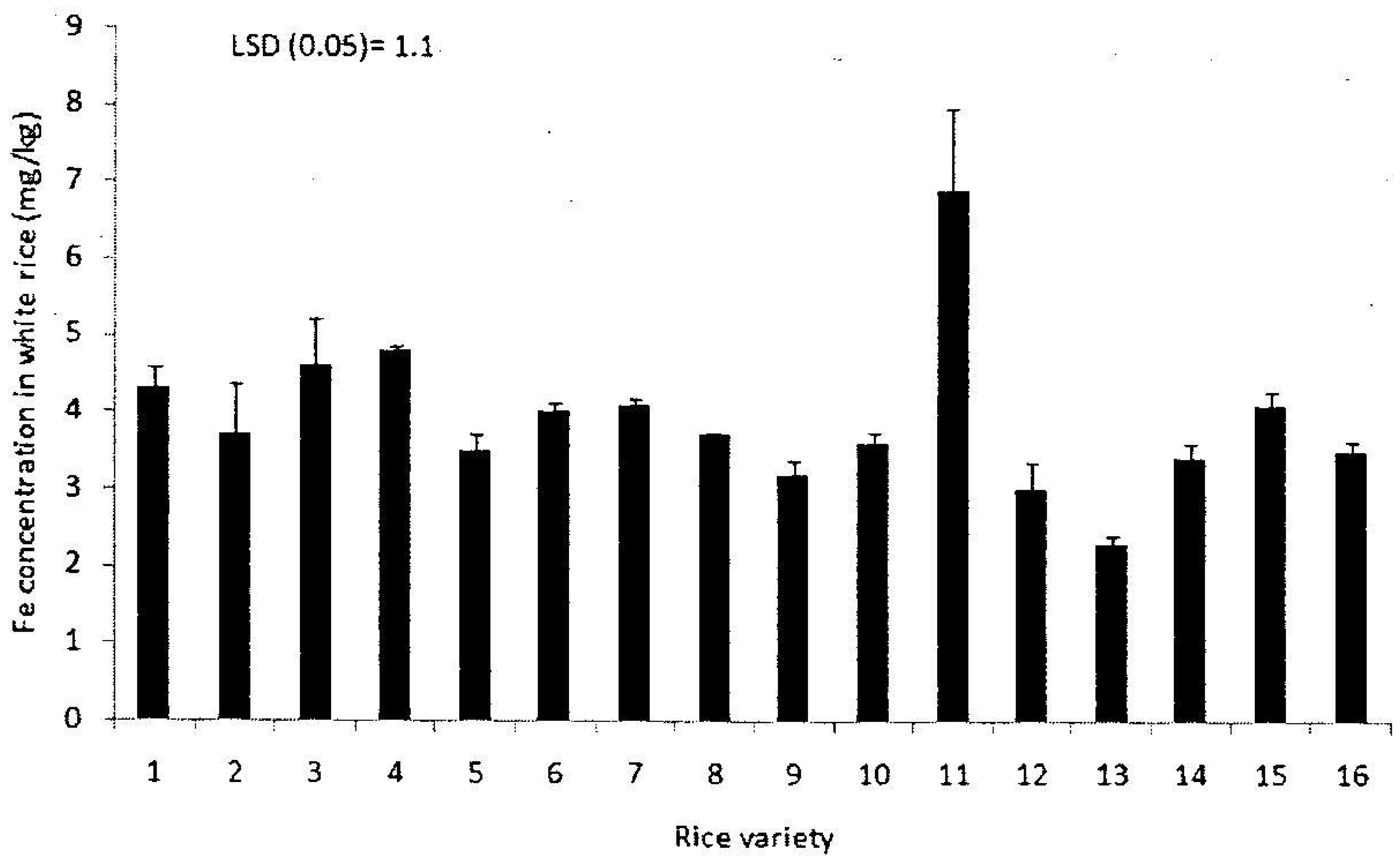
รูปที่ 1 ปริมาณสารแอนโทไซยานินในข้าวกล้องของข้าวทั้ง 16 พันธุ์ (mean ± SE) (n=3)



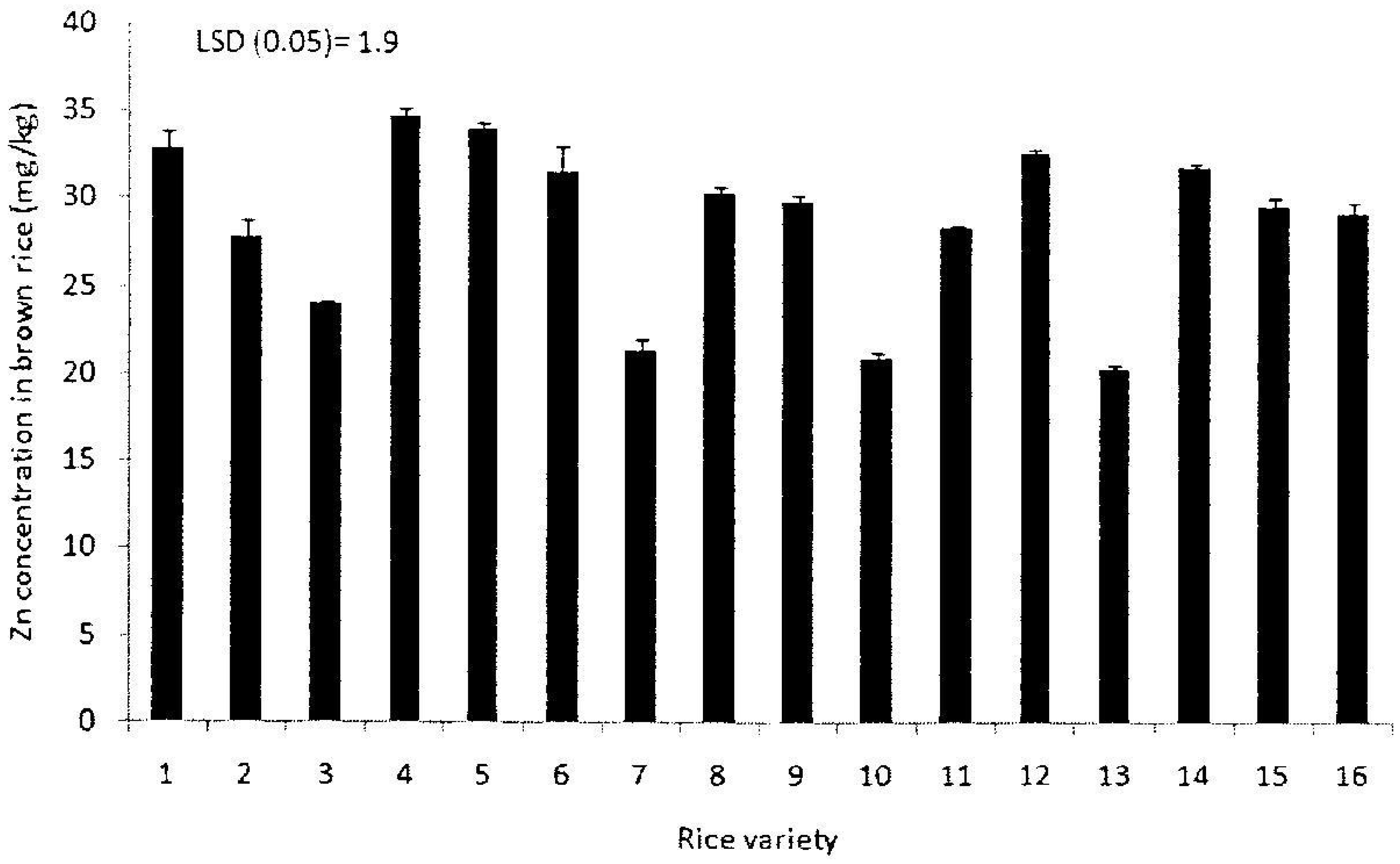
รูปที่ 2 ปริมาณสารแอนโทไซยานินในรำข้าวของข้าวทั้ง 16 พันธุ์ (mean ± SE) (n=3)



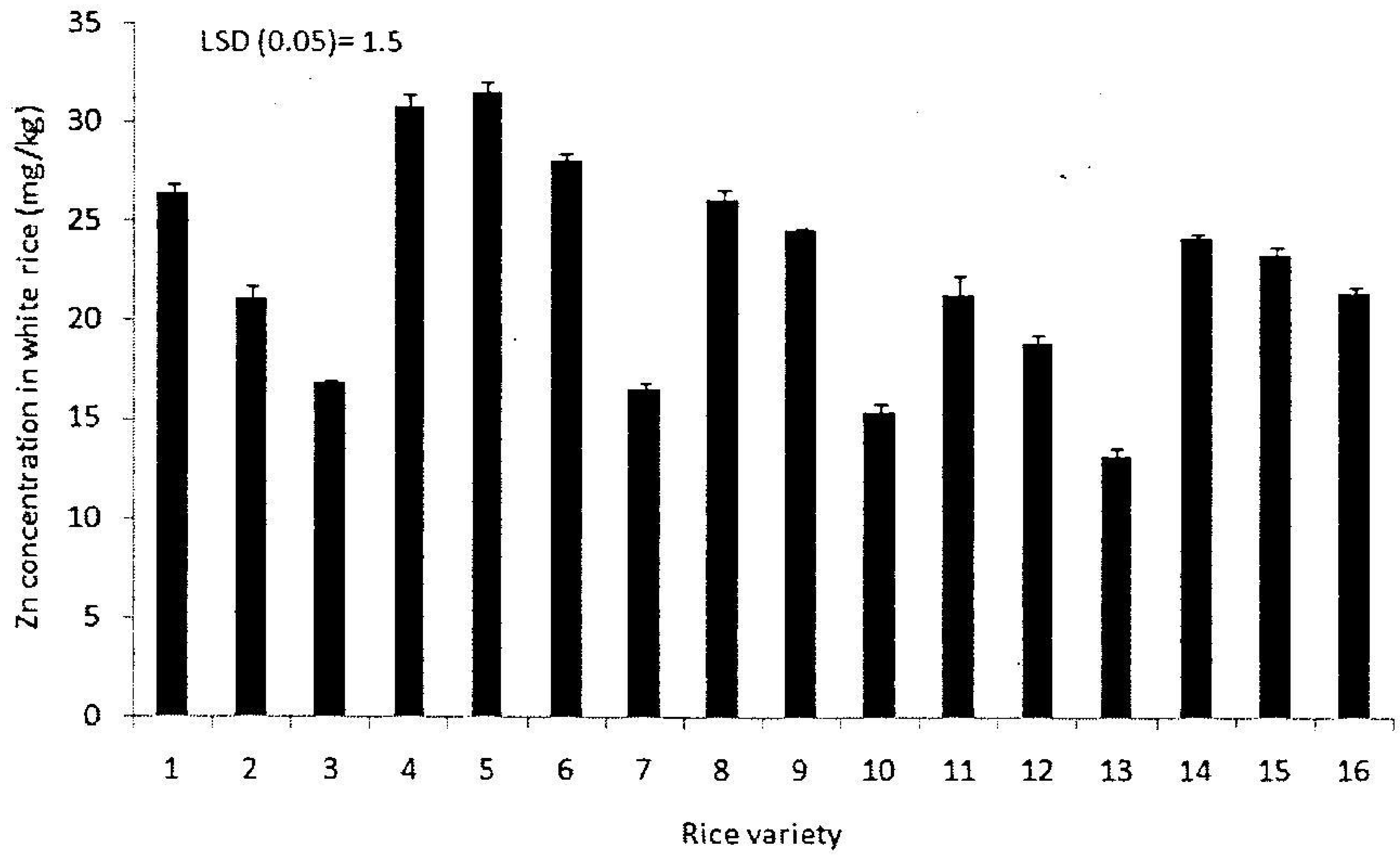
รูปที่ 3 ปริมาณธาตุเหล็กในข้าวกล้องของข้าวทั้ง 16 พันธุ์ (mean ± SE) (n=3)



รูปที่ 4 ปริมาณธาตุเหล็กในข้าวสารขัดขาวของข้าวทั้ง 16 พันธุ์ (mean ± SE) (n=3)



รูปที่ 5 ปริมาณธาตุสังกะสีในข้าวกล้องของข้าวทั้ง 16 พันธุ์ (mean ± SE) (n=3)



รูปที่ 6 ปริมาณธาตุสังกะสีในข้าวสารขัดขาวของข้าวทั้ง 16 พันธุ์ (mean ± SE) (n=3)

สรุปผลการทดลอง

การทดลองนี้บ่งชี้ว่ามีสารอาหารที่สำคัญ เช่นสารแอนโทไซยานิน ธาตุเหล็กและสังกะสี อยู่ในเมล็ดข้าวของข้าวเฉดสี โดยเฉพาะในส่วนของเนื้อเยื่อหุ้มเมล็ดที่มีสีดำ ซึ่งสามารถนำมาใช้ประโยชน์ในทางโภชนาการของผู้บริโภคข้าวได้ แต่อย่างไรก็ตามยังมีความแปรปรวนของสารแอนโทไซยานิน ธาตุเหล็กและสังกะสีในข้าวเฉดสีแต่ละพันธุ์ ซึ่งความแปรปรวนนี้อาจจะขึ้นอยู่กับลักษณะทางพันธุกรรม สภาพแวดล้อม และปฏิสัมพันธ์ของพันธุกรรมและสภาพแวดล้อมที่จะต้องมีการศึกษาและวิจัยถึงรายละเอียดต่อไป

คำขอบคุณ

ขอขอบคุณสำนักงานพัฒนาการวิจัยการเกษตร (สวก) องค์การมหาชน สำนักงานกองทุนสนับสนุนการวิจัย (สกว) และสำนักงานคณะกรรมการการอุดมศึกษาภายใต้โครงการมหาวิทยาลัยวิจัยแห่งชาติที่สนับสนุนงบประมาณในการวิจัย

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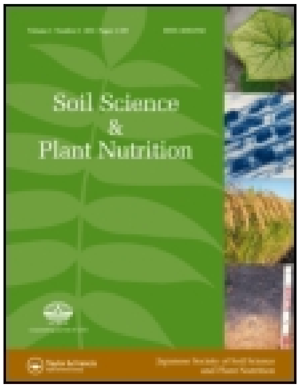

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Effect of different foliar zinc application at different growth stages on seed zinc concentration and its impact on seedling vigor in rice

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ORIGINAL ARTICLE

Effect of different foliar zinc application at different growth stages on seed zinc concentration and its impact on seedling vigor in rice

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Abstract

This study evaluated how zinc (Zn) concentration of rice (*Oryza sativa* L.) seed may be increased and subsequent seedling growth improved by foliar Zn application. Eight foliar Zn treatments of 0.5% zinc sulfate ($ZnSO_4 \cdot 7H_2O$) were applied to the rice plant at different growth stages. The resulting seeds were germinated to evaluate effects of seed Zn on seedling growth. Foliar Zn increased paddy Zn concentration only when applied after flowering, with larger increases when applications were repeated. The largest increases of up to ten-fold were in the husk, and smaller increases in brown rice Zn. In the first few days of germination, seedlings from seeds with 42 to 67 mg Zn kg^{-1} had longer roots and coleoptiles than those from seeds with 18 mg Zn kg^{-1}, but this effect disappeared later. The benefit of high seed Zn in seedling growth is also indicated by a positive correlation between Zn concentration in germinating seeds and the combined roots and shoot dry weight ($r = 0.55$, $p < 0.05$). Zinc in rice grains can be effectively raised by foliar Zn application after flowering, with a potential benefit of this to rice eaters indicated by up to 55% increases of brown rice Zn, and agronomically in more rapid early growth and establishment.

Key words: rice, foliar application, seed zinc, seedling growth, $ZnSO_4$.

INTRODUCTION

Zinc (Zn) is essential in protein synthesis and gene expression in plants (Cakmak 2000; Broadley *et al.* 2007). It has been estimated that about 10% of the proteins in biological systems need Zn for their structural and functional integrity (Andreini *et al.* 2006). This element has also been indicated to be required as a cofactor in over 300 enzymes (Coleman 1998). During germination, production of reactive oxygen species (ROS) is well known (Cakmak *et al.* 1993; Bailly *et al.* 2002; Qin and Liu 2010) and Zn plays a central role in detoxification of ROS in plant cells (Cakmak 2000; Broadley *et al.* 2007).

In addition to being essential to plants, Zn is also an essential mineral nutrient for human beings. It is estimated that 1/3 of the world population is affected by Zn deficiency that is associated with low dietary intake. Zinc deficiency is known to have serious adverse impacts on human heath, especially in children, such as impairments in physical growth, immune system, and learning ability, and causing DNA damage and cancer development (Keen and Gershwin 1990; Ho *et al.* 2003; Black *et al.* 2008). Increasing Zn concentration of stable food crops is, therefore, an important humanitarian challenge.

Cereals are the major source of Zn for the world's population, especially for the poor people living in rural areas. However, Zn contents of cereal-based foods are quite inadequate to meet human demands. The problem is especially acute for rice consumers as rice (*Oryza sativa* L.) has the lowest Zn content among the cereals

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(Juliano 1993). Considerable variation in brown rice Zn has been found among different rice genotypes. Ranges of 13.5 to 58.4 mg Zn kg^{-1} have been reported for a large rice germplasm at International Rice Research Institute (IRRI) which averaged only 25.4 mg Zn kg^{-1} compared with 35.0 mg Zn kg^{-1} in wheat (*Triticum aestivum* L.) (Welch and Graham 2002). In most cases, rice cultivated soils are very low in plant-available Zn leading to further decreases in grain Zn concentration (Alloway 2004; Cakmak 2008). Previous studies have reported that about 30% of the cultivated soils of the world are Zn deficient and about 50% of the soils used for cereal crop production have low levels of Zn available for plants (Sillanpaa 1982; Graham *et al.* 1992; Welch 1993; Cakmak 2002). Several strategies have been suggested to improve Zn concentration in rice seed, raising concentration up to 60 mg Zn kg^{-1} for agronomic benefits and human health, such as conventional breeding, fertilizer management, seed priming and fortification (Welch and Graham 2004; Cakmak 2008; Prom-u-thai *et al.* 2010). Rice genotypes are highly different in their tolerance to soil Zn deficiency (Hoffland *et al.* 2006; Wissuwa *et al.* 2006; Gao *et al.* 2009); but the role of this genetic variation for Zn deficiency tolerance in grain Zn accumulation is not well studied. In case of wheat, it seems that the genes affecting grain Zn accumulation and expression of Zn deficiency tolerance are different (Cakmak *et al.* 2004). As discussed by Cakmak *et al.* (2004), there is even an inverse relationship between high Zn-deficiency tolerance and grain Zn accumulation. Probably, grain Zn concentrations are diluted due to higher grain yield capacity of the genotypes showing higher tolerance to soil Zn deficiency.

Previous studies indicated that growing wheat crops with high seed Zn resulted in better seedling vigor and seed viability, higher yield, and lessening of seed rate required for sowing, especially on potentially Zn-deficient soils (Rengel and Graham 1995; Rengel 2002; Cakmak 2008). Applying Zn fertilizers in the soil also increases dry matter, grain yield and grain Zn concentration in rice (Shehu and Jamala 2010; Fageria *et al.* 2011). Sowing rice seeds with high Zn content (mixing seed in Zn powder) was found to result in a rapid and better germination, greater root length and better shoot growth (Slaton *et al.* 2001). Foliar spray of Zn represents an effective practice to improve grain Zn significantly. In wheat, foliar application of Zn fertilizer improved both productivity and grain Zn concentration up to three or four fold as a foliar spray (Cakmak 2008). In rice, foliar application of Zn significantly increased Zn accumulation in the grain (Yuan *et al.* 2013). Thus, enrichment of seeds with Zn benefits both crop production and health of the consumers, especially those whose Zn intake comes primarily from cereal grains.

When compared to soil applications, foliar Zn application is more effective in increasing grain Zn (Yilmaz *et al.* 1997; Cakmak *et al.* 2010a). Although foliar application of Zn is a promising method to increase seed Zn concentration, its effectiveness may also depend on several factors. One of these factors is the time of foliar Zn application. In the case of wheat it was shown that the highest Zn concentration in seed was achieved when foliar Zn was applied after the flowering stage (Zadok's scale 7; Zadok *et al.* 1974) compared to the applications realized before the flowering stage (Ozturk *et al.* 2006; Cakmak *et al.* 2010a).

In the case of rice, there is no published information on the effect of appropriate timing of foliar Zn application on Zn accumulation in the different forms of grain (paddy, brown and husk) and how the elevated level of seed Zn may consequently affect seedling growth. The present study has been conducted to determine the appropriate timing of foliar Zn application that would maximize seed Zn concentration and also consequently improve seedling growth.

MATERIALS AND METHODS

Foliar Zn application

The experiment was conducted at the demonstration research field of Chiang Mai University, Chiang Mai, Thailand in the wet season from June to November 2010. Rice seeds of cultivar CNT 1 were derived from Phitsanuloke Rice Research Center, Thailand. Seeds were sown on the seedbed and grown for 30 d. The soil used in this experiment contained 1.05 mg diethylenetriamine-pentaacetic acid (DPTA) extractable Zn kg^{-1}, 68.1 mg P kg^{-1} and the pH was 5.68. Seedlings were transplanted into the field plots with 25×25 cm between hills with a single seedling per hill. The experiment was laid out in a completely randomized design with three replications, in a total experimental area of 20×40 m. Basal nitrogen (N) fertilizer in the form of ammonium sulfate (NH_4SO_4) was applied at 30 d after transplanting at the rate of 100 kg N ha^{-1}. Zinc was applied by foliar application with 0.5% zinc sulfate ($ZnSO_4 \cdot 7H_2O$) solution at different stages during plant growth (Table 1). The solution was prepared by dissolving $ZnSO_4$ powder with triple distilled deionized (TDI) water. The prepared solution was poured into the sprayer. The foliar application was applied by evenly spraying the solution until the whole plants were wet and the solution just began to drip from leaves, in the morning around 10 am. The rate of application was 900–1000 L ha^{-1}. At maturity, grain yield and yield components (straw dry weight, tiller and panicle number per plant) were evaluated. Seed Zn concentration was analyzed in three

Table 1 Periods of foliar spray application with 0.5% zinc sulfate ($ZnSO_4 \cdot 7H_2O$) in rice (*Oryza sativa* L.) cultivar CNT 1 at different stages of plant growth. The foliar application was applied around 10 am by evenly spraying the solution until the whole plants were wet and solution just began to drip from leaves. The rate of application was 900–1000 L ha^{-1}

Treatment	Plant growth stage when foliar spray application with 0.5% $ZnSO_4 \cdot 7H_2O$	Symbol
1	Control (no foliar)	Nil
2	Panicle initiation	PI
3	Booting	BO
4	Panicle initiation and 1 week after flowering	PI + 1WAF†
5	1 week after flowering	1WAF
6	2 weeks after flowering	2WAF
7	1 week and 2 weeks after flowering	1 + 2WAF
8	Panicle initiation + Booting + 1 week and 2 weeks after flowering	PI + BO + 1 + 2WAF

†WAF = Week after flowering.

Table 2 Grain yield and yield components of rice (*Oryza sativa* L.) cultivar CNT 1 when foliar application with 0.5% zinc sulfate ($ZnSO_4 \cdot 7H_2O$) was applied at different stages of plant growth

Treatment	Grain weight (g $plant^{-1}$)	Straw dry weight (g $plant^{-1}$)	Plant dry weight (g $plant^{-1}$)	Plant height (cm)	Tillers $plant^{-1}$	Panicles $plant^{-1}$
1	19.4	27.1	48.3	67.7	15.0	13.0
2	20.0	34.6	54.6	69.1	18.0	12.0
3	19.3	22.3	41.6	67.8	16.0	13.0
4	18.5	26.9	45.3	69.8	15.0	12.0
5	20.3	30.1	50.4	68.3	18.0	13.0
6	23.7	30.8	54.5	69.6	17.0	14.0
7	20.9	29.2	50.1	68.1	17.0	13.0
8	20.1	30.0	48.7	68.2	17.0	13.0
F test	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*
$LSD_{0.05}$	6.8	9.7	15.9	5.1	5.0	4.0

ns, no significant difference between treatments at $P < 0.05$; $LSD_{0.05}$, least significant difference at $P < 0.05$.

different forms of rice seed: paddy (unhusked seed), brown rice (husk removed manually) and the husk (palea and lemma). The chemical analysis was done by the dry ashing method, followed by Atomic Absorption Spectrophotometer (AA) (Zarcinas *et al.* 1987).

Seedling growth and development

Rice seeds with three different Zn concentrations in paddy rice from the foliar Zn spray experiment described above (no foliar (Nil); two weeks after flowering (2WAF); and panicle initiation (PI) plus booting (BO) plus one week (1WAF) and two weeks after flowering) were sub-sampled and used to test their effectiveness on seedling growth. High, intermediate and low seed Zn concentrations selected were as follows: 67 mg Zn kg^{-1}, 42 mg Zn kg^{-1} and 18 mg Zn kg^{-1} respectively. The three groups of seeds were germinated on germination paper in plastic trays with 50 seeds per tray. Moisture was provided with deionized water. The experiment was conducted in three replications. The germination period was 7 d (D7). Root and coleoptile length were measured every day from day 0 (D0) to 7 (D7) and seedlings were also collected every day for Zn analysis. At D7, roots and coleoptiles were separated into three parts, root, coleoptiles and germinating seed, before being oven dried at 75°C for 72 h for measuring dry weight and Zn concentration following the method described above.

Data analysis

The data were subjected to analysis of variance (ANOVA) and means that were significantly different were separated at $P < 0.05$ by the least significant difference (LSD) test.

RESULTS

Effect of growth stage on yield and seed Zn concentration

Foliar application of Zn at various growth stages and frequencies had no effect on grain yield and yield components (Table 2), but had a significant effect on Zn concentration of the rice seed, analyzed as paddy (whole, un-husked), brown rice (the caryopsis with husk

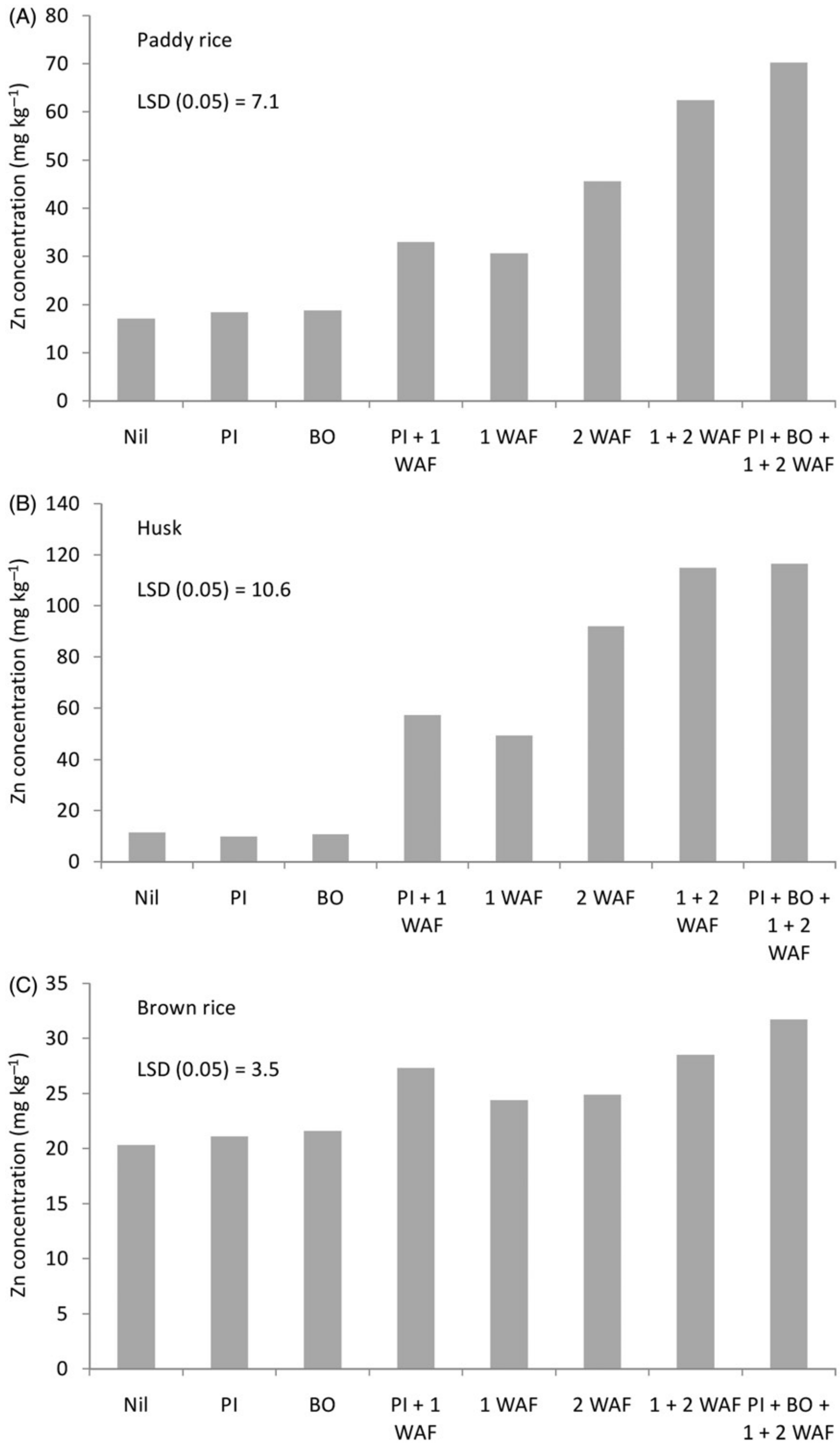

Figure 1 Zinc (Zn) concentration in paddy (A), husk (B) and brown rice (*Oryza sativa* L.) (C) of cultivar CNT 1 when foliar application with 0.5% zinc sulfate ($ZnSO_4$) was applied at different stages of plant growth. The data are mean of each Zn application treatment average from three independent replications. LSD (0.05), least significant difference at $P < 0.05$; Nil, no foliar; PI, panicle initiation, BO, booting; WAF, weeks after flowering.

removed) and husk (palea and lemma) (Fig. 1). In the Nil Zn treatment, there was 17 mg Zn kg^{-1} in the paddy, 20 mg Zn kg^{-1} in brown rice and 11 mg Zn kg^{-1} in the husk. Applying Zn before flowering, at panicle initiation or booting, had no effect on Zn concentration in all three components of the rice grain. The paddy Zn concentration was increased by 76% with one foliar Zn application at one week after flowering, by 164% at one week later, and by 264% when these two treatments were combined. The effects of timing and frequency of the Zn application were similar on Zn concentration of the rice husk and brown rice, but with major differences in the magnitude of increases. The foliar Zn treatments affected up to ten-fold increases in the husk Zn, and by 35%–55% in brown rice. In spite of the difference in magnitude of increases, the Zn concentration in brown rice was closely correlated to the Zn concentration in paddy ($r = 0.86$, $p < 0.01$) (Fig. 2).

Effect of seed Zn concentration on seedling growth

Seeds from the foliar treatments classed into three levels of Zn concentration, namely, low (18 mg Zn kg^{-1}), intermediate (42 mg Zn kg^{-1}) and high (67 mg Zn kg^{-1}), were found to have different impacts on seedling growth and change of Zn concentration in germinating seed. The concentration of seed Zn had clear effects on seedling growth as measured with the dry weight of roots and coleoptile (Fig. 3). There was a progressive increase in seedling weight with increasing seed Zn. High seed Zn also promoted root and shoot growth during the first few days of germination, although this effect disappeared later

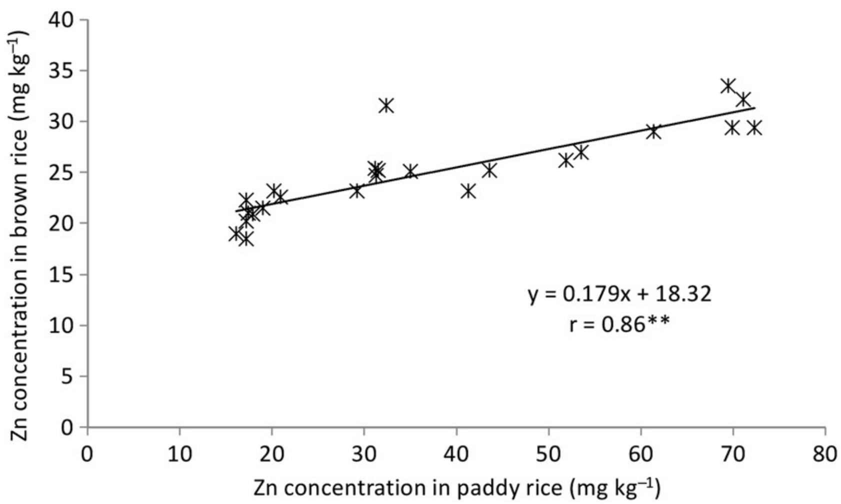

Figure 2 The relationship of zinc (Zn) concentration in paddy and brown rice (*Oryza sativa* L.) in cultivar CNT 1 when foliar application with different Zn treatments of 0.5% zinc sulfate ($ZnSO_4$) at different stages of plant growth ($n = 24$).

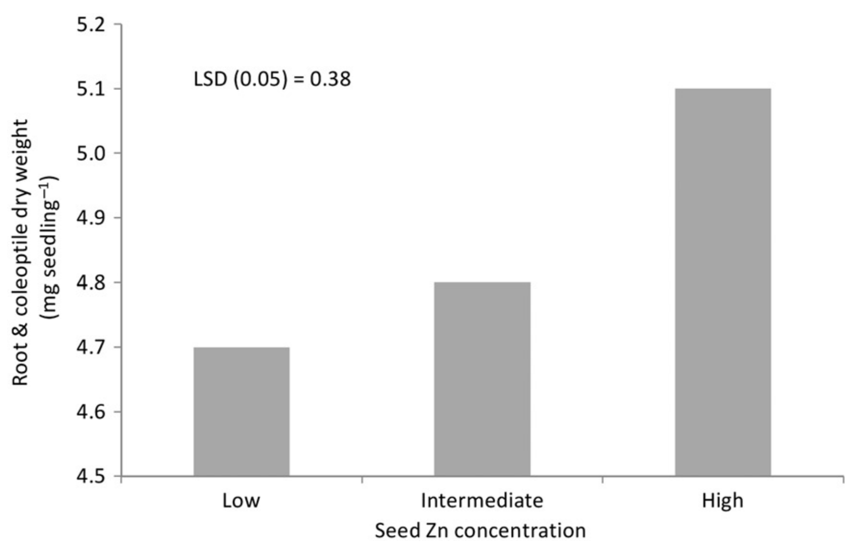

Figure 3 Root and coleoptiles dry weight of seven-day-old seedlings grown from rice (*Oryza sativa* L.) seed with high [67 mg zinc (Zn) kg^{-1}], intermediate (42 mg Zn kg^{-1}) and low (18 mg Zn kg^{-1}) Zn concentration. Each data point is mean of 3 independent replications. LSD (0.05), least significant difference at $P < 0.05$.

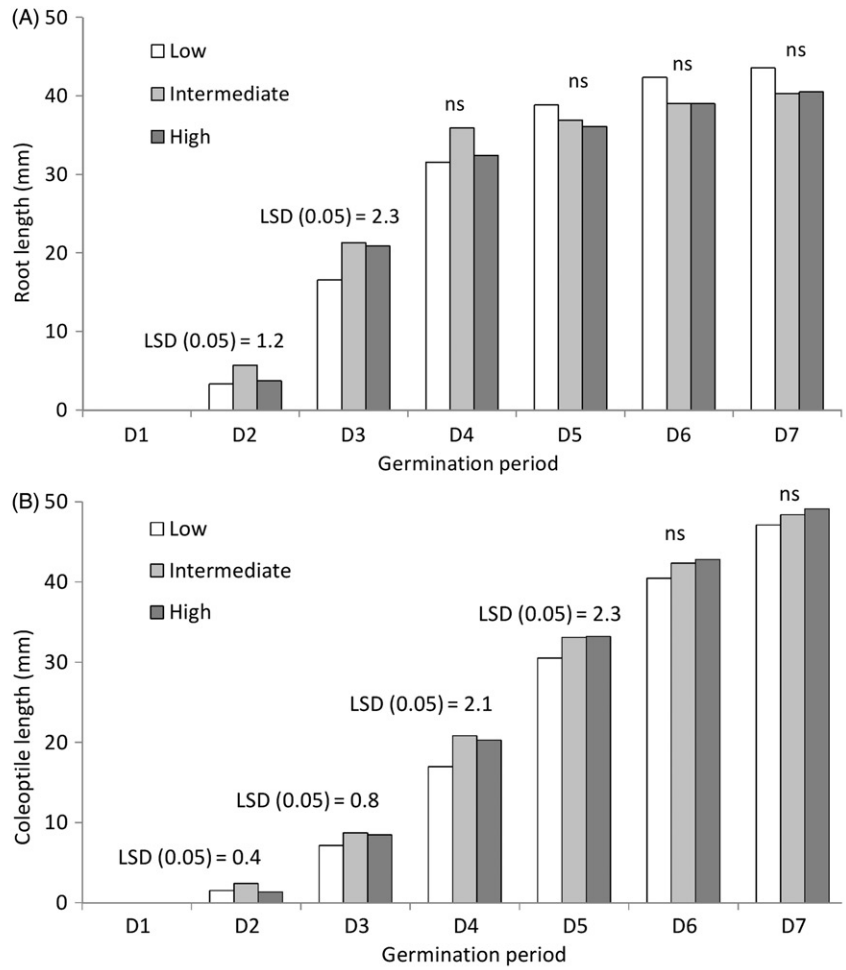

Figure 4 Root (A) and coleoptiles (B) length of seedling grown from rice (*Oryza sativa* L.) seed with high (67 mg Zn kg^{-1}), intermediate (42 mg Zn kg^{-1}) and low (18 mg Zn kg^{-1}) zinc (Zn) concentrations at each day from germination. Each data point is a mean of three independent replications. LSD (0.05), least significant difference at $P < 0.05$.

(Fig. 4). At D3, seedlings derived from seed with high and intermediate Zn had longer roots than those grown from seed with low Zn (Fig. 4A). Seedlings from low Zn seed also had shorter coleoptile than those from intermediate to high Zn seed until D5 (Fig. 4B). There was a positive correlation between seed Zn concentration and dry weight of root and shoot during germination period ($r = 0.55$, $P < 0.01$) (Fig. 5).

DISCUSSION

Foliar Zn application significantly increased Zn concentration in rice seed both in paddy and brown rice. This result is in good agreement with the previous studies in wheat in which seed Zn concentration was increased by foliar Zn application up to three times compared with no Zn application (Yilmaz *et al.* 1997; Cakmak *et al.* 2010a; Karim *et al.* 2012). Foliar Zn applied is easily absorbed and transported through phloem as shown in wheat by using radio labeled Zn (^{65}Zn), especially in plants grown under low Zn supply (Haslett *et al.* 2001; Erenoglu *et al.* 2002). Although xylem transport of Zn has been indicated to be more important for Zn accumulation in rice grain than re-translocation of Zn from the leaves (Palmgren *et al.* 2008), the results of this study, however, suggested that phloem transport of Zn from leaf and stem tissue may also play a greater role in enrichment of grains with Zn. In close agreement with the results presented in this paper, it has been shown very recently that soil-applied Zn fertilizers had very little effect on grain Zn in rice grown in five countries while spraying Zn to foliage caused very significant increases in grain Zn (Phattarakul *et al.* 2012). It seems that the growth conditions have an important role in contribution of xylem (root uptake) and phloem transport (remobilization) to grain Zn accumulation. Under conditions with

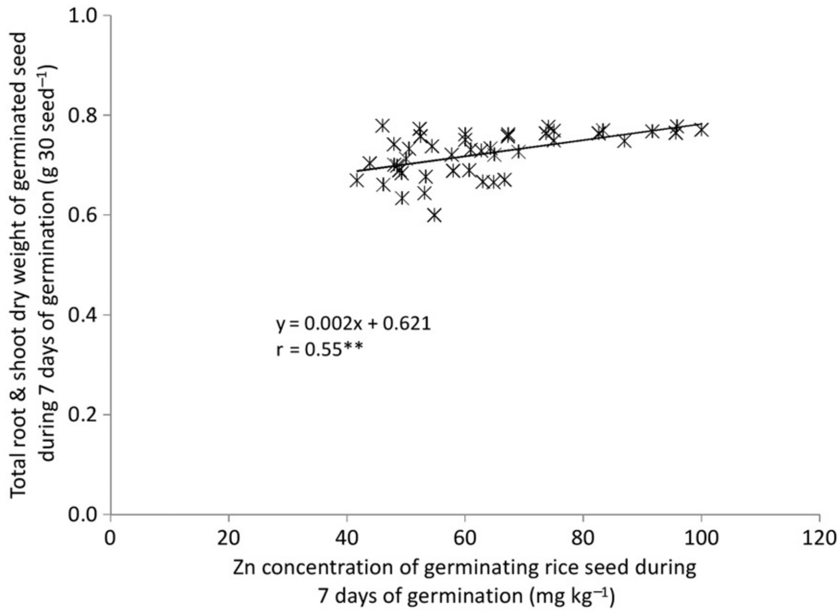

Figure 5 The relationship between zinc (Zn) concentration of germinating rice (*Oryza sativa* L.) seed and total shoot and root dry weight from three different seed Zn concentrations during seven days of the germination period.

complete Zn supply during the whole growth stage (e.g., greenhouse and growth chamber experiments) continuous root uptake and transportation of Zn into seeds would be a dominant pathway for Zn accumulation in grain. However, in the case of limited Zn supply to roots (e.g., dry field conditions), remobilization of Zn from the vegetative tissues into seeds would be a more relevant method for grain Zn accumulation (Waters and Grusak 2008; Cakmak *et al.* 2010a).

The present study has also shown that timing of the foliar application is an important factor in achieving the highest increases in grain Zn through foliar Zn spray. The highest Zn concentration in paddy rice was achieved with foliar Zn spray that was applied at four stages (panicle initiation, booting, 1 + 2 WAF), while foliar application of Zn at early growth stage such as at panicle initiation and/or booting had no effect on seed Zn concentration. There was an increase in brown rice Zn of up to 56% of Zn when Zn was applied after foliar Zn was applied at late growth stages (after flowering). It seems very obvious that only a small amount of foliar-applied Zn is translocated into paddy rice after early foliar application, while a greater amount of Zn is transported into paddy rice and penetrates into brown rice after late foliar application. This result is in close agreement with Phattarakul *et al.* (2012) who showed that a foliar Zn spray applied at late growth to rice grown under field conditions caused a greater increase in grain Zn than a foliar Zn spray before flowering stage. Similar results were also found in field grown-wheat (Cakmak *et al.* 2010a).

On the other hand, the present results contradict results previously reviewed by Stomph *et al.* (2009) that, unlike in wheat, the Zn loaded in rice grains was remobilized from roots, stem and sheaths but not from leaves. The present results together with those of Phattarakul *et al.* (2012) suggest that promoting the Zn content in vegetative tissues by foliar Zn application results in Zn being available for re-translocation into seeds. One of the reasons for the stimulated transport of Zn into seeds after the flowering stage might be related to significant increases in protein biosynthesis during the early stage of seed formation (Martre *et al.* 2003; Ozturk *et al.* 2006). As discussed by Cakmak *et al.* (2010b), increasing seed protein concentrations creates a sink for Zn, and there is a close positive correlation between seed protein and Zn concentrations. Also, having high Zn in the husk contributes greatly to grain Zn. The positive correlation between Zn concentrations of husk, paddy and brown rice ($r = 0.86$, $p < 0.05$) strongly supports the possibility that the husk represents one of the important sources of the re-translocated Zn into the rice grain. The vascular system connecting the husk with the caryopsis inside provides the conduit for photosynthates and nutrients; however, the pathway through which Zn in the husk is re-translocated to the rice grain inside needs to be further investigated. In wheat plants grown under field conditions, Zn is mainly transported into endosperm through the crease phloem (Cakmak *et al.* 2010a). Increases in Zn concentration of brown rice would provide important nutritional benefits to rice consumers, particularly for those who have limited access to Zn from other food sources. High grain Zn has also important agronomic benefits for plants grown under low Zn supply. In the present study, the agronomic benefit of high seed Zn in paddy rice was demonstrated by better seedling growth and development as compared with low seed Zn. High Zn in seeds may have diverse positive impacts on seedling development. One of the well-documented functions of Zn is related to its effect on protein synthesis and protein functions (Cakmak 2000; Broadley *et al.* 2007). There are nearly 2800 proteins which need Zn for their structural and functional integrity (Andreini *et al.* 2006). These findings indicate that there may be high need for Zn during root and coleoptile development, for active protein synthesis and/or other related functions. During the seed germination, production of reactive oxygen species (ROS) is unavoidable and seeds/seedlings have defense mechanisms against ROS production (Bailly *et al.* 2002; Qin and Liu 2010). One of the defense enzymes against ROS is superoxide dismutase which is Zn dependent (Cakmak 2000; Broadley *et al.* 2007).

In conclusion, the present study has shown that Zn in rice grain can be effectively raised by foliar Zn application, in particular when Zn was sprayed after flowering. Seeds with high Zn provide both agronomic and nutritional benefits. Since widespread occurrence of Zn deficiency in human populations is associated with low dietary Zn intake, special attention should be paid to foliar treatments of staple food crops with Zn. As shown in this paper for rice, this agronomic practice is highly effective and quick in increasing grain Zn.

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Variation of seed zinc in a local upland rice germplasm from Thailand

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Abstract

This study examined variation in seed zinc (Zn) in a local upland rice germplasm that may affect adaptation in a system of slash-and-burn. Individual seed Zn in farmers' seed lots of local upland rice varieties from a slash-and-burn system was evaluated by staining with dithizone (DTZ). Concentration of Zn in the farmers' seed lots and their single-seed descent genotypes grown at Chiang Mai University was determined by chemical analysis. The DTZ staining of individual seeds in most of the farmers' seed lots covered the intensity of standards that ranged from 19 mg Zn/kg (RD21) to 31 (Nam Roo) mg Zn/kg. Zinc content by chemical analysis was closely correlated with the weighted-average staining for each seed lot. Almost all of the single-seed descent genotypes had higher seed Zn than RD21; two-thirds were higher than Nam Roo. The variation within seed lots detected by DTZ staining was confirmed by seed Zn in the single-seed descent genotypes. Evolutionary adaptation to soil with limited Zn, exacerbated by alkalinity of the ash from slash-and-burn, is made possible by variation in seed Zn among individuals growing together in the same field, and benefits the eaters with Zn-enriched seed.

Keywords: adaptation; germplasm; seed; upland rice; zinc

Introduction

Seed zinc (Zn) content in food crops such as rice and wheat can have critical impact on the nutrition of the human population who depends on these grains for their staple, especially those with low income and those with limited access to costly Zn-rich food (Nestel *et al.*, 2006). Zn deficiency has serious adverse effects on human health and development in infants and children through impaired physical and mental growth (e.g. Keen and Gershwin, 1990; Black, 1998; Ho *et al.*, 2003). Considerable variation exists in seed Zn concentration among rice genotypes (Welch and Graham, 2002; Saenchai *et al.*, 2012), but seed Zn of some rice genotypes may differ significantly when grown in different soils (Graham *et al.*, 1999). The same rice variety grown on a soil with a high Zn supply could produce seed with up to twice as high Zn concentration of the rice grown on soil with low Zn supply (Phattarakul *et al.*, 2012). In soils where Zn is limited, rice genotypes with a high seed Zn content that can better survive and persist should also be beneficial to those dependent on them for staple.

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Local Thai rice varieties or landraces are recognized by the typical appearance of their plant and grain and unique key agronomic traits that describe their performance in the field or eating quality or both. In plants, adaptation is defined as genetic changes in populations that describe their evolutionary response (Lambers *et al.*, 1998). Under conditions of stress, such adaptation would be possible only where variation in the relevant traits enables the best fitted genotypes to be selected for. Analyses of functional traits as well as simple sequence repeat markers have demonstrated the possibility of evolutionary adaptation in local Thai rice germplasm (Pintasen *et al.*, 2007; Pusadee *et al.*, 2009; Oupkaew *et al.*, 2011). Most of the world's rice crop is grown as wetland crop, in which the soil is submerged under 10–20 cm of water for most of the growing season. Upland rice, the main staple crop for small farmers in many parts of tropical Asia (George *et al.*, 2001), is grown in well-drained soil, often on slopes. In Thailand and in other parts of Asia, upland rice is traditionally grown in a system of shifting cultivation or 'slash-and-burn', in which the vegetation is cut down, left to dry in the sun and then burned. Soil fertility is improved as nutrients stored in the biomass are released by burning, soil acidity neutralized or made more alkaline by the ash (Zinke *et al.*, 1978; Yimyam *et al.*, 2003). In ash pockets, the rice seed sown may encounter Zn deficiency more extremely than might be expected from a rise of one-half to one unit of pH of the bulk soil by burning. In such conditions, high seed Zn could be advantageous, especially during early establishment. This study examined variation in seed Zn in a local upland rice germplasm that may affect adaptation in a system of slash-and-burn in which a high seed Zn concentration may be advantageous, and also benefits eaters of the rice.

Materials and methods

Seed samples of five local rice varieties, namely Pa Ai Khupe (PA), Bue Bang (BB), Bue Gua (BG), Bue Mue Tabong (BM) and Bue Polo (BP), were obtained from farmers in Huai Tee Cha (19°78′ N, 93°84′ E; altitude 900 m), Sob Moei District, Mae Hong Son Province, about 250 km southwest of Chiang Mai (Fig. 1(a)). There were four seed lots each from different farmers of PA and BM, five seed lots of BG and BP, and two seed lots of BB. The village field from which the rice seeds were produced was on a clay loam to loam soil. Soil fertility characteristics determined on ten replicated samples to 30 cm depth included pH (1:1, soil:water), organic matter (Walkley and Black), total nitrogen (Kjeldahl), available phosphorus (Bray II), extractable potassium and Diethylenetriaminepentaacetic acid (DTPA)-extracted Zn. The seed Zn content was determined on seed without husk.

Experiment 1: Detecting variation in seed Zn with dithizone staining

The Zn content was determined in individual 20 seeds of 15 seed lots in four local rice varieties (four of PA and BG, two of BB and five of BP) by dithizone (DTZ) staining (Ozturk *et al.*, 2006), and in the bulk seed of each of the seed lots in triplicates using an atomic absorption spectrophotometer (Allan, 1961). After the husk was removed by hand, the rice seed was soaked for 3 h in Distilled De-Ionized water (DDI) water, cut into half lengthwise through the embryo using a Teflon-coated razor blade to avoid contamination during separation (Prom-u-thai *et al.*, 2003), then placed in freshly prepared DTZ (500 mg 1,5-diphenyl thiocarbazone (Merck) in 1 l of AR grade methanol) (Prom-u-thai *et al.*, 2010). Samples were rinsed thoroughly in DDI water and blotted dry. The intensity of the red colour staining of the endosperm on the internal surface of the cut grain was rated at three levels: 1, least intense; 3, moderately intense; 5, most intense, compared with low (RD21) and high (Nam Roo) Zn standards, under an Olympus DP12 optical microscope (Tokyo, Japan) (Fig. 1(b)). The weighted-average staining for each seed lot, the DTZ index, was computed by the formula:

$$\text{DTZ index} = \sum[1p(1) + 3p(3) + 5p(5)],$$

where $p(1)$, $p(2)$, $p(3)$ = proportion of seed with 1, 3 and 5 DTZ staining, respectively. For example, if the proportion of the seed with 1, 3 and 5 DTZ staining is 11/20, 9/20 and 0/20, respectively, then

$$\text{DTZ index} = \sum[1(11/20) + 3(9/20) + 5(0/20)] = 1.9.$$

To determine Zn in the bulk seed lots, subsamples of the seed without husk (containing 40–50 seeds) were dried at 70°C for 72 h, and dry-ashed in a muffle furnace at 535°C for 8 h, the ash dissolved in 1:1 HCl, and Zn is determined with an atomic absorption spectrophotometer (Allan, 1961).

Experiment 2: Variation in seed Zn within and among seed lots

Ten single-seed descent genotypes were produced from four farmers' seed lots each from PA and BM, five seed lots each from BG and BP and two seed lots each from BB. The genotypes were grown together in wetland (water level kept at 10–15 cm above the soil surface

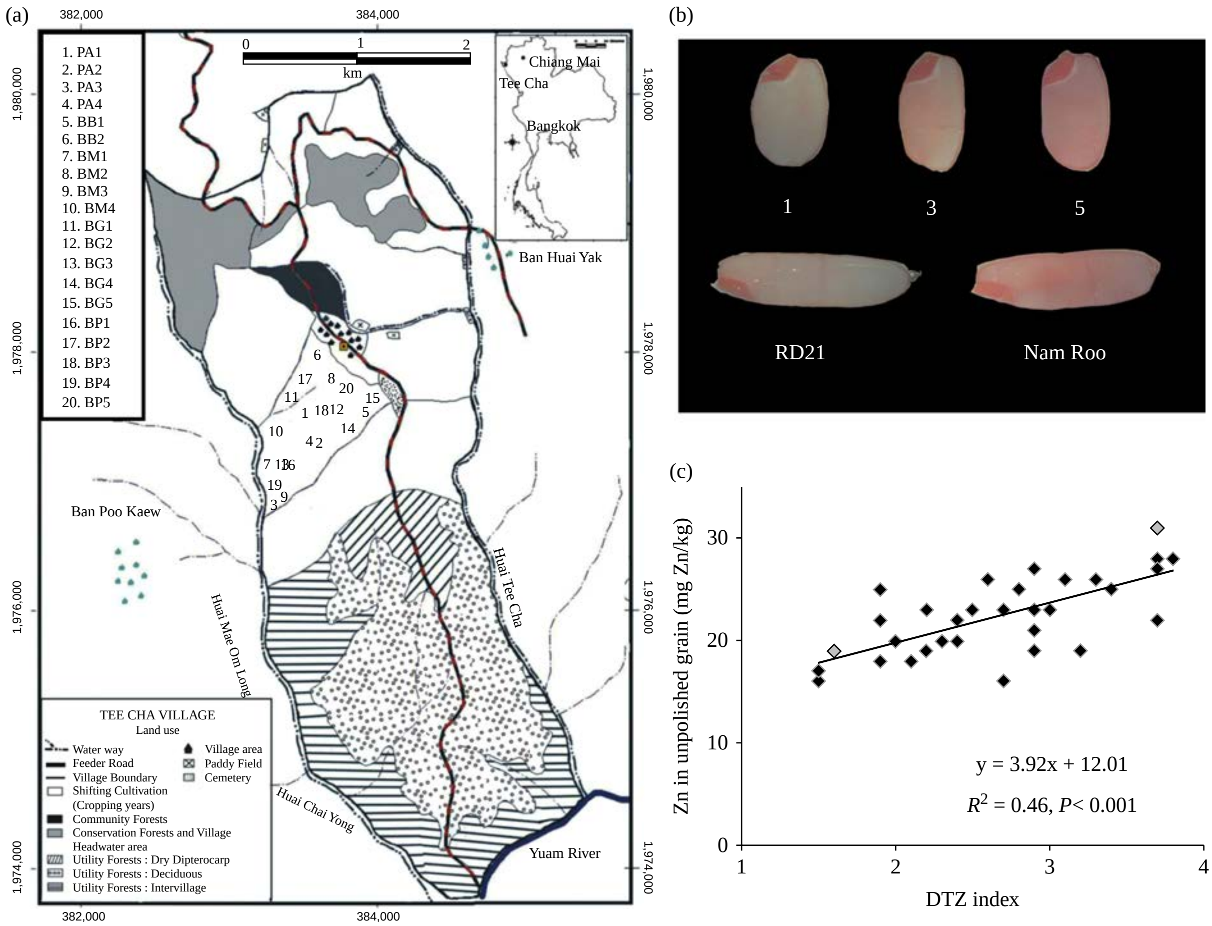

Fig. 1. (a) Location of different seed lots from farmers in Huai Tee Cha, Sob Moei District, Mae Hong Son province, Thailand; (b) three levels of DTZ staining intensity of unpolished rice grain (upper: 1, least intense; 3, moderately intense; 5, most intense) compared with standards (lower: RD21, 19 mg Zn/kg and Nam Roo, 31 mg Zn/kg); and (c) relationship between Zn in unpolished grain of rice from farmers' seed lots of four rice varieties (◆) and DTZ index with low (RD21) and high (Nam Roo) Zn checks (◈). Each point is the mean of three replicates, and the significance of linear regression was done by the analysis of variance.

until 3 weeks before harvest) and upland (irrigated and allowed to drain) conditions on a sandy loam soil at Chiang Mai University (CMU), Thailand. Plants were grown at 0.25 × 0.25 m spacing, in single 5-m rows for each genotype. Soil pH, available P and DTPA extract Zn of the soil were determined as for the field soil. The crops were fertilized with 68 kg N, 25 kg P and 25 kg K/ha. The two standard low and high Zn varieties

Table 1. Soil fertility characteristics of the field at Tee Cha village that provided the farmers' upland rice seed and at CMU for evaluation of progeny lines

Soil fertility characteristic*	Tee Cha	CMU
Texture	Clay loam to loam	Sandy loam
pH (1:1, soil:water)	4.6	5.5
% Organic matter (Walkley and Black)	3.9	nd
% Total N (Kjeldahl)	0.9	nd
P (mg/kg) (Bray II)	2.9	59.0
K (mg/kg) (sodium acetate extraction)	140	nd
Zn (mg/kg) (DTPA extraction)	0.4	1.5

nd, not determined.
* Values are means of ten replications.

Table 2. Analysis of variance in the seed without husk of five local upland rice varieties in different seed lots from farmers' seed store

Source	*F*-test	*P*
Variety (V)	55.10	<0.001
Seed lot (S)	31.27	<0.001
V × S	7.95	<0.001

(RD21 and Nam Roo) were included in the planting for comparison. After determination of seed yield at maturity, subsamples of seed without husk were analysed in triplicate for Zn as in Experiment 1.

Results

The soil at Huai Tee Cha where the farmers' seed was produced was acidic with only 0.4 mg DTPA-extractable Zn/kg and 2.9 mg available P/kg compared with 1.5 mg DTPA-extractable Zn/kg and 59 mg available P/kg in the soil in the experimental field at CMU (Table 1). The seed Zn of upland rice from the farmers' field, ranged from 16 to 28 mg Zn/kg, with a significant difference among varieties, seed lots and their interaction (Tables 2 and 3). The range of within-variety variations in seed Zn of the upland rice from farmers' field was especially large in PA and BP, with 16–18% coefficient of variation (CV).

The intensity of DTZ staining clearly differentiated between the low and high Zn standards, RD21 with 19 mg Zn/kg and Nam Roo with 31 mg Zn/kg (Fig. 1(b)). This qualitative measure of seed Zn concentration was made quantitative by the weighted-average staining for each seed lot, the DTZ index, which was closely associated with Zn concentration by chemical analysis that averaged the Zn content over 40–50 seeds (Fig. 1(c)).

Variation in seed Zn content within each of the farmers' seed lots was indicated by variation in the intensity of DTZ staining (Fig. 2(a)). Individuals in the majority of seed lots showed DTZ staining intensity that ranged from least intense of RD21 (1), to most intense of Nam Roo (5) and intermediate between the two extremes (3).

The variation in seed Zn content within seed lots indicated by DTZ staining of individual seeds was confirmed by the seed Zn determined chemically from their single-seed descent genotypes grown as an upland and wetland crop at CMU, with 10% or higher CV of the seed Zn in half of the seed lots (Table 4) and frequency distribution of the genotypes by their seed Zn covering three or more classes of Zn concentration in two-thirds of the seed lots of upland crop (Fig. 2(b)). This high level of variation in seed Zn was found in all of the upland rice varieties examined. When grown together in the same field, almost all of the single-seed descent genotypes developed from the farmers' upland rice germplasm had a higher concentration of seed Zn than RD21, the low Zn standard, while two-thirds of the genotypes had as high as or higher seed Zn than Nam Roo, the high Zn standard. In spite of the variation, the seed Zn of single-seed descent genotypes developed from each seed lot was closely associated with the seed Zn of the original seed lot from farmers' field (Fig. 3(a)). The seed Zn of the genotypes grown as a wetland crop was also closely associated with their seed Zn when grown as an upland crop (Fig. 3(b)). The variation in seed Zn in this germplasm did not appear to have resulted from dilution effects, and the association between seed Zn and seed yield was very weak for both upland ($R^2 = 0.04$) and wetland ($R^2 = 0.05$) grown seed (Fig. 3(c)).

Discussion

In spite of the low soil Zn of only 0.4 mg DTPA-extractable Zn/kg, most of the rice produced and consumed in this village was rich in Zn than common varieties produced in the lowlands for urban consumers, which were grown on soils with a much higher

Table 3. Zn concentration in the seed without husk of five local upland rice varieties in different seed lots from farmers' seed store

Variety	Seed lot	Seed Zn (mg Zn/kg)	Variety	Seed lot	Seed Zn (mg Zn/kg)
PA	1	22abc	BG	1	23abc
	2	20ab		2	28c
	3	25bc		3	27bc
	4	16a		4	25bc
BB	1	28c		5	27bc
	2	26bc	BP	1	23abc
BM	1	24bc		2	23abc
	2	25bc		3	25bc
	3	27bc		4	16a
	4	24bc		5	22abc

Different small letters indicate significant differences ($P \leq 0.05$; Duncan's multiple range test).

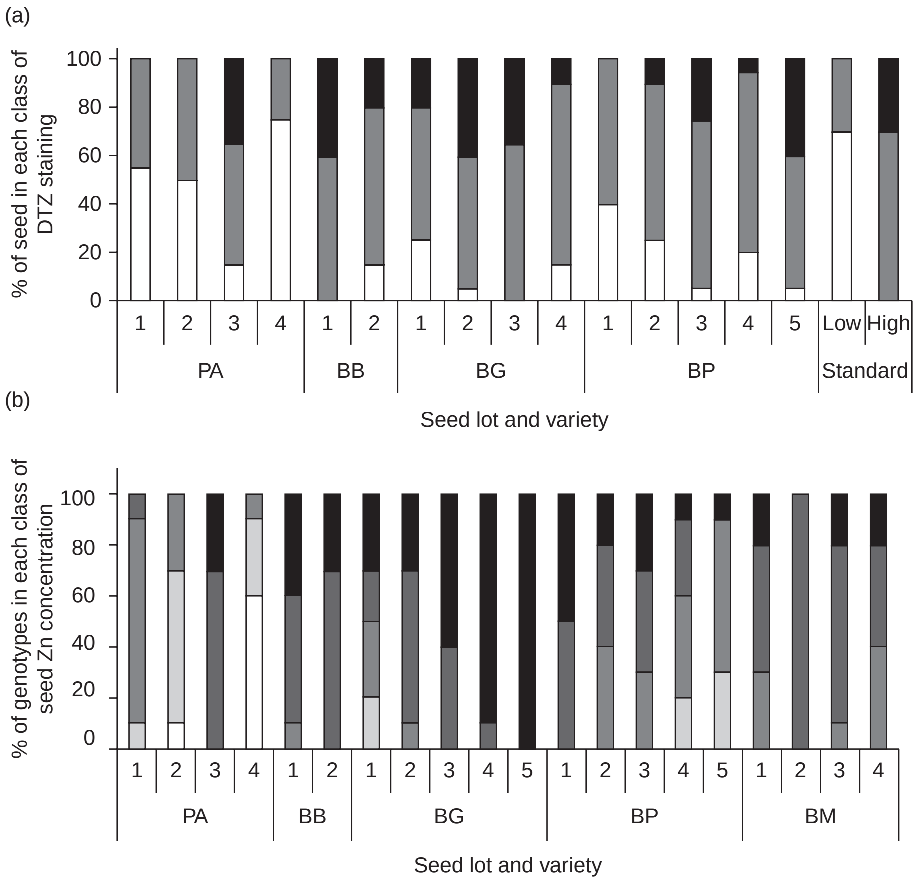

Fig. 2. (a) Distribution of individual seeds (20 seeds per sample) from different seed lots of four local rice varieties of an upland crop with low (RD21, 19 mg Zn/kg) and high (Nam Roo with 31 mg Zn/kg) Zn standards, by DTZ staining intensity in three classes (1 = least intense, □; 3 = moderately intense, ■; 5 = most intense, ■); and (b) distribution of single-seed descent genotypes from different seed lots of five local rice varieties by the Zn concentration of their unpolished grain in five classes (mg Zn/kg: <20, □;21–25, ■; 26–30, ■; 31–35, ■; >35, ■).

available Zn. Unpolished grain of rice from China, India and Thailand produced from popular modern varieties of each country on soils with 2.1–5.5 mg DTPA-extractable Zn/kg had only 21 ± 3 mg Zn/kg (Phattarakul *et al.*, 2012). The same study also showed that a high yielding Thai rice variety CNT1 produced grain with only 10 mg Zn/kg on a soil with 0.5 mg DTPA-Zn, while the grain contained 20 mg Zn/kg when grown on a soil with 2.1 mg DTPA-Zn.

Staining Zn with DTZ has been effectively used to rank wheat genotypes by their seed Zn concentration as well as to study the localization of Zn in different tissues of wheat seed (Ozturk *et al.*, 2006). The very high Zn concentration in the rice embryo, shown to range from 119 to 254 mg Zn/kg and up to 17 times that of the endosperm (Saenchai *et al.*, 2012), was highlighted by its most intense staining in this study. The Zn content by chemical analysis was positively correlated with the weighted-average staining for each seed lot. The DTZ staining indicated the variation in individual seeds within seed lots and these were confirmed by the seed Zn determined chemically from their single-seed descent genotypes grown as an upland crop at CMU. Similar variation among individual seeds within seed lots including those with completely uniform appearance was seen in seed Fe with Perl's Prussian blue staining, confirmed with chemical analysis (Pintasen *et al.*, 2007).

Zinc requirement of germinating seed and early seedling growth may be met externally by Zn in the soil or applied directly to the seed (Slaton *et al.*, 2001; Prom-u-thai *et al.*, 2012; Boonchuay *et al.*, 2013) as well as internally from the Zn stored within the seed (Rengel and Graham, 1995; Cakmak, 2008). The ability to accumulate Zn in the seed at very high concentration (Cakmak *et al.*, 2000, 2004) would have been a key adaptation trait where the external Zn supply is limited, for example, in the wild and primitive wheat grown within the centre of diversity of wheat with extremely low Zn in the soil such as the Central Anatolia Region of Turkey (Cakmak *et al.*, 1996). The molecular basis of rice explained that *OsZIP4* was induced and expressed under Zn deficient in the root apical

Table 4. Zn concentration in the seed without husk (mean, coefficient of variation and range) of individual plants grown as an upland and wetland crop at CMU from different seed lots of five local rice varieties

		Upland			Wetland		
		Seed Zn (mg Zn/kg)			Seed Zn (mg Zn/kg)		
Variety	Seed lot	Mean	%CV	Range	Mean	%CV	Range
PA	1	29	5.8	25–31	32	8.5	30–39
	2	24	9.9	20–27	28	4.6	26–30
	3	35	6.3	31–38	34	10.8	25–37
	4	21	14.2	17–27	27	10.1	23–31
BB	1	35	9.5	29–39	32	14.5	21–37
	2	34	5.6	31–36	34	9.0	29–36
BM	1	32	10.1	28–39	34	10.0	30–41
	2	33	3.4	31–35	36	11.0	31–45
	3	34	10.0	29–41	37	8.0	32–44
	4	33	16.9	27–44	39	4.2	36–41
BG	1	31	21.3	22–40	35	22.0	20–49
	2	34	8.0	30–38	36	8.0	29–40
	3	36	5.7	33–39	36	8.3	31–42
	4	38	8.9	31–45	37	5.3	34–40
	5	38	3.5	36–40	40	5.0	37–43
BP	1	36	6.7	31–39	41	14.0	32–50
	2	33	11.4	27–39	37	5.1	30–47
	3	32	11.7	27–38	30	13.0	23–35
	4	29	16.1	23–38	32	10.7	27–37
	5	28	15.6	23–38	31	11.9	25–39

meristem and vascular bundles (Ishimaru *et al.*, 2007). The evolutionary adaptation of rice variety with high seed Zn thus involves this mechanism. In the system of slash-and-burn cropping, the seed of upland rice is sown into the ash-covered soil in which there will be extremely alkaline pockets. The availability of Zn in the soil is depressed by alkalinity (Marschner, 1993), thus external Zn supply for early growth and establishment of the upland rice would be limiting, especially in soils already very low in available Zn. The adaptation advantage in such an environment would be with those genotypes that can accumulate more Zn in their seed. We have shown here how this was indeed the case with a local rice germplasm developed under slash-and-burn on a low Zn soil (0.4 mg DTPA-extractable Zn/kg), with two-thirds of the genotypes having seed Zn as high as or higher than the high Zn standard Nam Roo when grown on a soil where Zn was less limited (1.5 mg DTPA-extractable Zn/kg). The high Zn standard Nam Roo was in fact developed from a local upland rice variety of the same name from Nam Roo, a highland Lisu village (BRRD, 2014) some 130 km north of Chiang Mai. The stability ranking of seed Zn among rice varieties was shown from the positive relationship

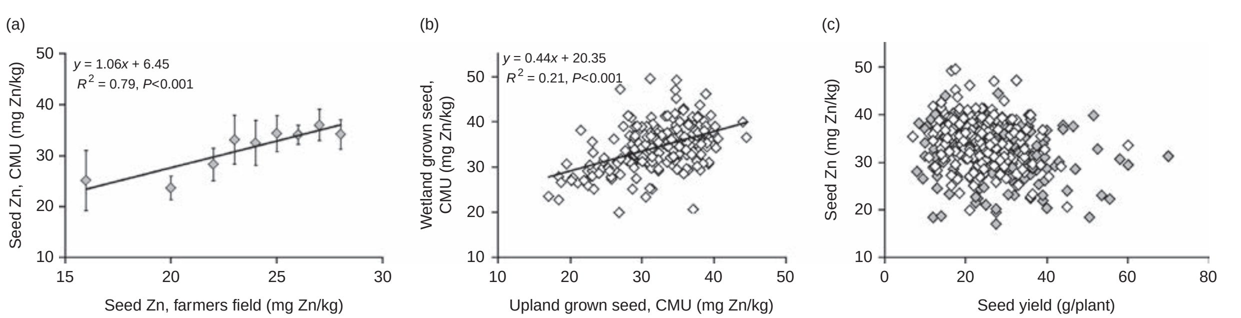

Fig. 3. Relationship (a) between seed Zn of upland rice seed lots from farmers' field and of single-seed descent genotypes developed from farmers' seed lots (mean ± SD) grown as an upland crop in experimental field at CMU, and (b) between seed Zn of the genotypes grown as wetland and upland crops at CMU; (c) relationship between seed Zn and seed yield of single-seed descent genotypes of upland rice grown as upland (◆, $R^2 = 0.04$) and wetland (◇, $R^2 = 0.05$) crops at CMU. Significance of linear regression was done by the analysis of variance.

between Tee Cha village and CMU, even though the difference in seed Zn of the same variety was observed between the two locations. This suggested that environmental condition plays an important effect on seed Zn in rice such as growing location.

Local crop varieties or landraces are recognized for their potential to adapt to local conditions and changing farming practices and environments (Frankel *et al.*, 1995; McCouch, 2004). Adaptation of plants to stress is an evolutionary process (Lambers *et al.*, 1998). Continuing evolution of domesticated species is a central tenet of *in situ* or on-farm conservation (e.g. Brown, 1999; Brush, 1999), recognized by the Conference on Biological Diversity (United Nations, 1992). Such evolutionary adaptation to specific stresses is, however, only possible when there is variation in the key traits to enable the most fit in the population to be selected for. Molecular diversity in rice has been demonstrated in numerous studies, including those that focus on diversity within populations and varieties (e.g. Saini *et al.*, 2004; Zhu *et al.*, 2004; Pusadee *et al.*, 2009; Choudhury *et al.*, 2013). There are, however, few studies of within-population diversity in functional traits. The high value of CV within seed lot of the same variety from each farmer's field could be explained by (Ceccarelli and Grando, 1999; Rerkasem, 2008). The first is due to the variation among individuals growing and completing their life cycle in the same field. This could be explained by the basis conscious and unconscious on-farm selection that have resulted in agronomically important adaptation and tolerance to stress. Farmer's selection is greatly aided by conspicuous variations among individuals. Selection for glutinous or non-glutinous rice, preferred staple of different ethnic groups, is made by their readily distinguishable waxy (opaque) and non-waxy (translucent) endosperm type. From a gall midge-resistant glutinous local variety, gall midge-resistant non-glutinous rice has been developed by farmers who prefer non-glutinous rice for staple, living at higher elevations where the gall midge is emerging as a new threat (Oupkaew *et al.*, 2011; Chaksan, 2013).

The prevalence of rice genotypes with high seed Zn in this local upland rice germplasm indicated that a high concentration of Zn in the seed was a key adaptation trait for rice in a slash-and-burn system on a low Zn soil, which also benefitted the people who grow and eat this rice. Variation in seed Zn among individuals in each seed lot suggested a continuing process of evolutionary adaptation, and also highlighted the need to focus on individuals in the population when evaluating such germplasm for potentially useful traits that would not be detectable by precise quantitative assessment of the phenotype like the chemical Zn analysis that averages over 40–50 individuals.

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Full Length Article

Effect of Polishing Time on Distribution of Monomeric Anthocyanin, Iron and Zinc Content in Different Grain Layers of Four Thai Purple Rice Varieties

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Abstract

This study aimed to establish how distribution of nutrients might vary in surface grain layers of purple rice by evaluating their monomeric anthocyanin, Fe and Zn concentration affected by polishing. Concentration of monomeric anthocyanin, Fe and Zn, grain dimension and pigmentation intensity of 4 purple rice varieties (KD, high monomeric anthocyanin; KH, high Fe; LP, high Zn- monomeric anthocyanin; BI, low monomeric anthocyanin-Fe-Zn) from Thailand was determined after polishing five times. Pigmentation reduced in all varieties after 15 s polishing, while monomeric anthocyanin concentration declined in BI and KD but increased in KH and LP. Monomeric anthocyanin ultimately declined in all varieties with longer polishing, but there was no correlation between monomeric anthocyanin concentration and pigmentation intensity at 0 to 30 s. Micronutrients Fe and Zn concentration was increasingly depressed with longer polishing, although the effect on Zn was relatively less. Monomeric anthocyanin, Fe and Zn were distributed differently in successive layers of rice grain removed by polishing, with major varietal difference for monomeric anthocyanin. In some varieties, highest monomeric anthocyanin concentration was in the outer-most grain layer, but was richest below the surface in others. The highest concentration of Fe and Zn were also in outer-most layer, although more Zn was distributed deep in endosperm and most of Fe was removed after 30 s. Such variation should be taken into consideration in efforts to retain or recover potentially valuable nutrients from the grain of purple rice. © 2015 Friends Science Publishers

Keywords: Monomeric anthocyanin; Iron; *Oryza sativa*; Purple rice; Zinc

Introduction

Rice with pigmented pericarp is a common element of Asia's rice culture. Varieties with various shades of purple are called 'purple' or 'black' rice to differentiate from ordinary, non-pigmented rice with colorless or off-white pericarp. Those with reddish pericarp, not to be confused with 'red rice' contamination of grain with sub-standard quality from weedy or wild rice, are somewhat rarer. Reports on black and purple rice varieties have come from all over Asia (Appa Rao *et al*., 2006; Ahuja *et al*., 2007). The Asian purple rice is predominantly of the waxy endosperm type (Chaudhary and Tran, 2001; Appa Rao *et al*., 2006), which become sticky and glutinous rice when cooked. Pigmented rice has long been part of Asia's traditional pharmacopoeias. In Korea, black rice is considered a health food (Park *et al*., 2008). Pigmented rice is used in Chinese traditional medicine to prevent anemia and improve blood circulation, kidney function and eyesight (Deng *et al*., 2013). Porridge made from black rice is given to aid recovery of invalids; one Chinese variety is known as "healing of broken bones" (Chaudhary, 2003). Claims of medicinal properties of purple rice in Thailand include stopping bleeding after childbirth, bringing down fever to curing skin disease and diarrhea (Kaladee, 2011).

Purple rice is known under local name of *Khao Kam* (dark colored rice) in Lao PDR (Appa Rao *et al*., 2006) and northern Thailand, for the dark color of its unpolished 'brown rice', de-husked caryopsis, with embryo, pericarp and aleurone still intact, the form normally used. Nutritional and pharmacological properties of purple rice have been elucidated in many reports focusing on its content of many nutrients including compounds with capacity to neutralize the toxic effects of oxygen radicals, or antioxidative property. Although some of these studies were based on analysis of extracts from brown rice (Park *et al*., 2008; Yodmanee *et al*., 2011), others (Han *et al*., 2004; Saenjum, 2012) have clearly shown that most compounds of

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interest are in the 'bran' section, surface layers of de-husked caryopsis (pericarp, the aleurone and some sub-aleurone cells) plus the embryo which are removed by polishing in the milling process. Removal of bran by polishing or buffing to produce 'white rice' generally preferred by rice eaters has been clearly shown to depress Fe and Zn concentration (Prom-u-thai *et al*., 2007; Saenchai *et al*., 2012).

Although, pigmented rice has traditionally been used for preparation of deserts and special foods for festivities and religious offerings, and rarely consumed as staple, there is now increasing interest in it as a delicacy and as a source of various nutrients and compounds with medicinal and pharmacological potential. Numerous modern high yielding black rice varieties have been developed in China (Chaudhary, 2003). In Thailand, where special quality characteristics continue to be discovered in local purple rice landraces and new varieties developed (Prom-u-thai and Rerkasem, 2001; Phengrat and Jearakongman, 2009; Kaladee, 2011; BRRD, 2013), purple rice is a common part of the country's retail rice market, with prices many times higher of ordinary rice. The objective of this study was to evaluate how concentration of monomeric anthocyanin, Fe and Zn are affected by different intensity of polishing, and to shed light on localization of nutrients in successive grain layers of four different purple rice varieties. These results may help in milling and processing to modify and optimize recovery or retention of potentially useful novel compounds.

Materials and Methods

Plant Culture

Four purple rice varieties (Kam Hom Morchor, KH; Kam Doi Saket, KD; Luem Pua, LP; Bieisu, BI) were grown together in field in wet season (June-November), 2012, at Chiang Mai University, Thailand. The soil was sandy loam of Sansai series. Rice seeds were soaked in water overnight and incubated moist until germinated and grown for 30 days in a seedbed. Single seedlings were transplanted into hills at 25 × 25 cm spacing. Nitrogen fertilizer was applied using 75 kg N ha^{-1}, half at maximum tillering and half at flowering. The field was kept flooded under 0.1 – 0.2 m of water until maturity. Rice seed was harvested at maturity.

Sample Preparation

The rice seed was de-husked to produce unpolished rice (0 s) with a laboratory husker (Model P-1 from Ngek Seng Huat Co. Ltd., Thailand). Three replicates of 50 g sub-samples of unpolished rice of each variety were milled separately at 15, 30, 45 and 60 s with a laboratory polisher (Model K-1 from Ngek Seng Huat Co. Ltd., Thailand). The metal parts of both de-husker and polisher were carefully cleaned to avoid Fe and Zn contamination between samples. Grain dimension (width × length × thickness) were measured on fifty grain from each sample of 0, 15, 30, 45 and 60 s polished rice with a digital caliper, putative volume of the grain was derived by multiplication of grain width, length and thickness, to determine the extent of grain surface removal by different degree of polishing. Pigmentation intensity was measured with a chroma meter (model CR-300, Minolta, Osaka, Japan), with the *L* value representing degree of light or darkness of sample ($L = 100$, light; $L = 0$, dark). Whiteness was evaluated in a milling meter (Satake, Japan).

Chemical Analysis

Samples were oven dried at 75°C for 72 h before Fe and Zn determined using a Hitachi Z-8230 atomic absorption spectrophotometer (Zarcinas *et al*., 1987). Monomeric anthocyanin was determined by the pH-differential method (Escribano-Bailón *et al*., 2004; Giusti and Wrolstad, 2001). Briefly, 2.5 g freeze dried samples were extracted in double deionized water (DDI) at 50°C for 30 min. The extracted solution was filtered with filter paper before preparing two dilutions. Volume of one was adjusted with potassium chloride buffer (pH 1.0), and the other with sodium acetate buffer (pH 4.5). Each dilution was allowed to equilibrate for 15 min. Absorbance of the first dilution was measured at 520 and second at 700 nm, against a blank cell filled with distilled water. Absorbance readings were made after 15 min and completed within 45 min. The absorbance of the diluted sample (A) was calculated as follows:

$$A = (A_{520}-A_{700})_{pH1.0}-(A_{520}-A_{700})_{pH4.5}$$

The monomeric anthocyanin pigment concentration in the original sample was determined with the following formula:

$$\text{Monomeric anthocyanin content (mg L}^{-1}) = (A \times MW \times DF \times 1000)/(\varepsilon \times 1)$$

The concentration was converted to mg of total anthocyanin content /100 g sample (MW, molecular weight of cyanidin-3-glucoside = 449.2, DF = dilution factor; ε, molar absorptivity of the pigment = 26,900).

Data Analysis

The data were subjected to analysis of variance (ANOVA) and means were separated at $P< 0.05$ by the least significant difference (LSD) test. Certain sets of data were also subjected to correlation and regression analysis.

Results

Unpolished (0 s) grain of four rice varieties differed significantly in nutrient contents, intensity of pigmentation and dimension (Table 1). These purple rice varieties ranged from moderately high to high Fe and Zn, with KH being highest in Fe and LP highest in Zn. Monomeric anthocyanin concentration of unpolished rice grain was highest in KD, slightly lower in LP, much lower in KH and very little in BI.

Table 1: Dimension of unpolished grain (0 s) of 4 purple rice varieties, their iron, zinc and anthocyanin content and degree of pigmentation

Attribute	Variety†			
	KH	KD	LP	BI
Grain dimension (width × length × thickness = putative volume)				
Width (mm)	3.14 d	2.63 b	2.86 c	2.51 a
Length (mm)	6.01 a	6.48 c	7.82 d	6.21 b
Thickness (mm)	2.04 d	1.77 a	1.89 c	1.83 b
Putative volume (mm^3)	38.40 b	30.10 a	42.20 c	28.50 a
Nutrient concentration‡				
Iron (mg Fe kg^{-1})	40.00 c	18.00 b	19.00 b	14.00 a
Zinc (mg Zn kg^{-1})	38.00 a	35.00 a	55.00 b	34.00 a
Anthocyanin (mg 100 g^{-1})	5.70 b	18.90 d	15.70 c	1.30 a
Degree of pigmentation§				
L value	25.60 a	35.20 b	39.20 c	50.80 d
Whiteness (%)	5.10 a	5.80 a	6.10 a	8.50 b

†Different letters indicate significant difference (by LSD at P <0.05) between variety within each row (n = 3);‡On dry weight basis;§The *L* value was measured with a chroma meter, representing grain brightness, ranging from 0 (dark) to 100 (light). Whiteness was measured with milling meter

Table 2: Changes in putative grain volume and iron, zinc and anthocyanin concentration by polishing in 4 purple rice varieties

Variety (V)	Polishing time (T)				
	15 s	30 s	45 s	60 s	Mean
Putative volume depressed (%)†					
KH	13.70	28.20	28.70	31.20	25.50 A
KD	15.70	27.60	31.00	33.50	27.00 A
LP	23.00	33.60	37.10	40.90	33.60 B
BI	16.80	23.50	27.60	29.60	24.40 A
Mean	17.30 A	28.30 B	31.10 BC	33.80 C	
Significant effects‡§: V, $P < 0.001$; T, $P < 0.001$; Not significant $P < 0.05$					
Zinc concentration depressed (%)†					
KH	15.20	31.30	27.90	31.20	27.50 B
KD	17.10	18.50	20.10	23.80	18.30 A
LP	19.10	18.20	21.70	22.70	20.20 AB
BI	8.10	15.60	19.80	15.80	14.80 A
	14.10 A	20.90 A	22.40 B	23.40 B	
Significant effects‡§: V, $P < 0.01$; T, $P < 0.01$; V×T Not significant $P < 0.05$					
Iron concentration depressed (%)†					
KH	31.30 aA	87.40 bB	94.80 cC	97.60 cB	77.80
KD	48.30 aC	66.30 bA	85.30 cB	87.20 cA	71.80
LP	37.10 aAB	69.20 bA	76.50 bA	89.00 cA	67.90
BI	42.50 aBC	68.80 bA	83.60 cB	88.50 cA	70.90
Mean	39.80	72.90	85.10	90.60	
Significant effects‡§: V, $P < 0.001$; T, $P < 0.001$; V × T, $P < 0.001$					
Anthocyanin concentration depressed (%)†					
KH	-148.70 aA	47.70 bB	83.90 bcA	91.80 cA	18.70
KD	57.90 aC	73.70 aB	87.60 aA	99.60 bB	79.70
LP	-43.20 aB	23.40 bA	72.30 cA	90.60 cA	35.80
BI	90.50 aC	100.00 bC	100.00 bB	100.00 bB	97.60
Mean	-10.90	61.20	86.00	95.50	
Significant effects‡: V, $P < 0.001$; T, $P < 0.001$; V × T, $P < 0.001$					

†Compared with 0 s. Significant difference by $LSD_{p<0.05}$ (of the arcsine values where relevant) designated by different lowercase letters in the same row and uppercase letter in the same column;‡By analysis of variance §After arcsine transformation

Measured by the *L* value of unpolished grain, the variety KH had darkest color, followed by KD, LP and BI, respectively. The *L* value was closely associated with whiteness (r = 0.95, P < 0.01), although KH was significantly less white and other 3 varieties were relatively less distinguishable when measured in whiteness. Monomeric anthocyanin concentration of 0 s rice did not correlate significantly (P < 0.05) with intensity of pigmentation, measured either in the *L* value or whiteness.

In spite of significant difference in the original grain dimension, putative volume of rice varieties was depressed to similar extent by polishing (Table 2). Most of the depression in grain volume occurred after 30 s, 17% at 15 s and 28% at 30 s, but only a little more with longer polishing. Polishing affected Fe, Zn and monomeric anthocyanin concentration and putative volume of grain in different rice varieties differently (Fig. 1). Differential effects of polishing on the nutrients can be seen in changes of their concentration over polishing time (Table 2). Concentration of both Fe and Zn declined with longer polishing, but the effect on Zn was much milder than on Fe. Without significant difference among the varieties (P< 0.05), the biggest Zn depression was 15% at 15 s, followed by another 7% decline at 30 s, and with little further effect of longer polishing. After 60 s, the grain Zn concentration still averaged three quarters of unpolished grain. Iron concentration was depressed more strongly, with sharper decline in high Fe variety KH, which was brought to the same level as in other 3 varieties after 30 s, and by 60 s polishing almost all of Fe removed in all 4 varieties (Fig. 1).

Monomeric anthocyanin concentration was affected by polishing differently from Fe and Zn, with different effects among the rice varieties. After 15 s, monomeric anthocyanin concentration was depressed by 58% in KD, 90% in BI but increased by 43% in LP and 145% in KH (Table 2). Variation in the effect of polishing on monomeric anthocyanin of the rice varieties continued to 30 s, when 76% of original concentration still retained in LP, compared with 52% in KH, only 26% in KD and none in BI. Relative richness in monomeric anthocyanin of rice varieties was thus markedly altered by polishing, with KD, the variety with the highest monomeric anthocyanin at 0 s, becoming the second lowest after 15 - 30 s. Intensity of pigmentation became increasingly faded with longer polishing time, as indicated by increasing *L* value, but at varying rates among the rice varieties (Fig. 2). The fading was most rapid in KH, the variety which had the most intense pigmentation at 0 s became the least dark after 60 s. Although there was a general trend of declining anthocyanin concentration with fading pigmentation over longer polishing, intensity of pigmentation of 0 – 30 s rice did not correlate significantly ($P < 0.05$) with monomeric anthocyanin concentration.

Discussion

Anthocyanin is considered the primary bioactive compound in purple rice (Hiemori *et al.*, 2009; Deng *et al.*, 2013). Purple rice varieties from southern Thailand were found to contain more than ten times the concentration of anthocyanin in reddish brown varieties (Yodmanee *et al.*,

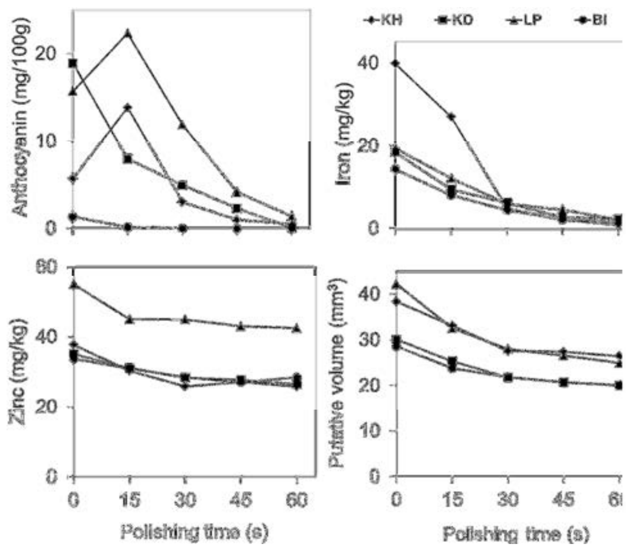

Fig. 1: Effect of polishing on anthocyanin (upper left), iron (upper right) and zinc (lower left) concentration and putative grain volume (lower right, derived from multiplication of grain width, length and thickness) in 4 purple rice varieties. (n = 3)

Significant effects by analysis of variance ($LSD_{p<0.05}$ in brackets)

	Variety (V)	Polishing time (T)	V×T
Zinc	$P < 0.001$ (1.4)	$P < 0.001$ (1.5)	$P < 0.05$ (3.1)
Iron	$P < 0.001$ (1.3)	$P < 0.001$ (1.4)	$P < 0.001$ (2.9)
Anthocyanin	$P < 0.001$ (1.1)	$P < 0.001$ (1.2)	$P < 0.001$ (2.4)
Putative volume	$P < 0.001$ (0.9)	$P < 0.001$ (1.0)	$P < 0.001$ (1.9)

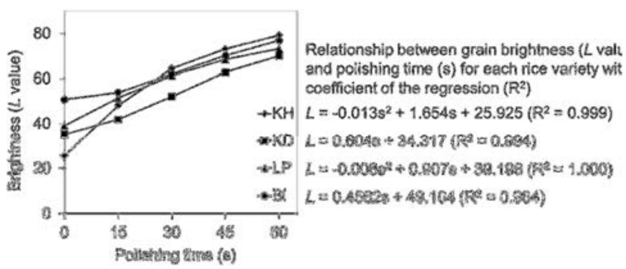

Fig. 2: Fading intensity of pigmentation by longer polishing, measured as degree of brightness, the *L* value on a chroma meter, ranging from 0 (dark) to 100 (light) in 4 purple rice varieties. (n = 3)

Significant effects by analysis of variance ($LSD_{p<0.05}$ in brackets)

Variety (V)	Polishing time (T)	V×T
$P < 0.001$ (1.0)	$P < 0.001$ (1.1)	$P < 0.001$ (2.2)

2011). This study has shown that monomeric anthocyanin concentration cannot always be predicted from intensity of pigmentation of the unpolished and lightly polished rice grain. In other plant species, lack of correlation between monomeric anthocyanin content and optical quality has been explained in different ways. For example, the amount of red light reflected from anthocyanin rich senescing leaves, and hence intensity of their red pigmentation was reported to be more dependent on distribution and content of chlorophyll instead of anthocyanin (Gould, 2004). As anthocyanin in red grapes and young wines disappears during fermentation and aging, the deep color characteristic of aged red wines has been shown to be the product of new wine pigments derived from complexes of monomeric anthocyanin with other molecules, especially with tannin (He *et al*., 2012; Brouillard *et al*., 2003). Pigmented rice varieties are generally very rich in tannin, with concentration in the bran 40-80 times than in bran of non-pigmented rice (Goffman and Bergman, 2004). Although unpolished grain of some Thai purple rice varieties have high in both monomeric anthocyanin and polyphenols (Yodmanee *et al*., 2011), it remains to be explored if complexes of anthocyanin with tannin or other phenols are part of the explanation of very low anthocyanin content of some purple rice varieties as found here.

Compared with published values for non-pigmented rice from other rice growing countries of Asia as well as Thailand (Saenchai *et al*., 2012), unpolished grain of the purple rice in this study ranged from moderately high to high Fe and Zn, with KH being the highest in Fe and LP highest in Zn. Polishing depressed Fe and Zn concentration in these purple rice varieties as reported for non-pigmented rice. The 30 s polishing produces regular milled rice in popular non-pigmented Thai rice varieties depresses the concentration of Fe by 46±12% and Zn by 31±15% (Saenchai *et al*., 2012). The sharp decline in Fe concentration after 30 s polishing indicated that Fe was more concentrated in surface layer of purple rice grain, especially the high Fe variety KH, with 87% decline of grain Fe after 30 s. Zinc in purple rice was distributed deeper in the endosperm, in the same way as the higher Zn varieties of non-pigmented rice, with Zn concentration of the purple rice depressed by only 21±7% at 30 s and 23±6% at 60 s. A previous study on milling depression in variety KD also investigated that anthocyanin, γ-oryzanol, and γ-tocopherol decreased by 74.49, 55.35, and 70.36%, respectively, after 10 s of milling (Laokuldilok *et al*., 2013). However, this study was carried out with an only variety (KD), which would be a variation of decreasing among different rice varieties.

The pattern of monomeric anthocyanin distribution found in the varieties KD and BI, with the highest concentration in outer-most grain layer, was somewhat similar to Fe and Zn. In these purple rice varieties, monomeric anthocyanin concentration was increasingly depressed with longer polishing. However, a different pattern of monomeric anthocyanin distribution was indicated with significant elevation of monomeric anthocyanin concentration by 15 s polishing in KH and LP varieties. This varietal difference in monomeric anthocyanin distribution was not related to concentration in unpolished grain, may possibly influence pigmentation intensity and optical property of purple rice. In non-pigmented rice, the form of grain preferred by consumer depend on white rice is produced by removal of bran. But for pigmented rice, bran may need to be considered in its different component tissues which include the pericarp, the aleurone layer, sub-aleurone cells and the embryo. The pericarp and embryo, components of the bran removed at 15 s constituted 17% depression of the putative volume, apparently contained little monomeric anthocyanin. In addition to monomeric anthocyanin, the bran of pigmented rice also contains other novel and potentially useful compounds, e.g. anti-oxidative phenols (Goffman and Bergman, 2004) and γ-oryzanol (Juliano *et al.,* 2005). Efforts to exploit such novel nutritional and pharmaceutical quality in pigmented rice, either in evaluation and screening of genotypes or in processing, may benefit from understanding of their distribution in the grain which could be affected by the milling process.

Conclusion

This study indicated that anthocyanin, Fe and Zn were distributed differently in successive layers of rice grain removed by polishing, with major varietal difference for the anthocyanin. In some varieties the highest anthocyanin concentration was in the outer-most grain layer, but was richest below the surface in others. The highest concentration of Fe and Zn were also in outer-most layer, although more Zn was distributed deep in the endosperm and most of Fe was removed after 30 s. These results could help in milling and processing to modify and optimize recovery or retention of potentially useful novel compounds.

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