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GENERATION MEAN ANALYSIS OF LEAF BRONZING ASSOCIATED WITH IRON TOXICTY IN RICE SEEDLINGS USING DIGITAL IMAGING METHODS

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SUMMARY

The effectiveness of rice germplasm utilization in breeding programs for improving iron toxicity tolerance requires an understanding of their genetics mode. Here, we study estimating gene effect using digital imaging method for iron toxicity tolerance in rice seedling. The proportion of intensity of color red (R)/green (G) index was analyzed by Adobe Photoshop CS3©. Validation was done using 23 genotypes of rice seedling under 400 mg.L^{-1} Fe^{2+} using Yoshida-agar solution. A genetics study was done using the six-generation population of 2 crosses between 2 iron toxicity tolerant varieties with an iron-toxicity sensitive variety (Inpara 5 x Mahsuri and Inpara 5 x Pokkali). The seedlings of parents and their progeny were phenotyped under same iron concentration for screening above. The simple additive-dominance which did not fit to the model indicated the presence of non-allelic gene interactions or epistasis in all observed traits. Five parameter models, additive x additive (i), additive x dominance (j) and dominance x dominance (l) epistasis, in addition to additive (d) and dominance (h) were fit for gene action explanation of the observed traits in both populations. These type of gene actions were duplicate epistasis in both crosses, except for R/G in cross of Inpara 5/Pokkali. R/G index showed strong correlation with LBS, shoot length, and root length in the F_2 population. Estimated broad and narrow-sense heritability of observed traits were medium to high and medium to low, respectively.

Key words: Epistasis, gene action, quantitative inheritance, heritability

Key findings: R/G index can be used for quantifying leaf bronzing score in rice associated with iron toxicity and applicable for genetics study analysis. The R/G index was quantitatively inherited with complex gene action, therefore postponed selection in latter generation would be effective for development tolerance rice to iron toxicity.

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INTRODUCTION

Iron toxicity is one of the major constraints of rice production of irrigated-lowland in tropical countries. The typical soil of this areas is mostly formed with iron oxide mineral, while during submerged would create an excess of Fe^{2+} concentration in soil solution (Ponamperuma, 1972). Increase of iron uptake by root plant would lead to a physiological disorder of rice

plant involving over production of reactive oxygen species through Fenton reaction (Winterbourn, 1995). The common symptom when plant is affected by high amount of iron is a leaf bronzing, brownish-red spots which start from tips of lower leaves and spread to the basal parts (Dobermann and Fairhurst, 2000). Moreover, it could cause growth reduction and in some acute cases, may cause death of leaves of the whole plant (Fageria *et al.,* 2008). The best way to overcome the problem of iron toxic soils is to grow tolerant rice varieties (Audebert and Sahrawat, 2000). So far the tolerant varieties have been identified mostly are traditional varieties which tend to have low productivity (Ismail *et al.,* 2007). Hence, introducing the iron toxicity tolerant trait into high-yielding modern varieties is one approach for increasing rice productivity in this area.

The improvement of rice for iron toxicity needs an efficient and effective breeding program. The genetics studies provide information to the breeder for deciding the methods and mode of selection (Fehr, 1987). Several genetics studies on iron tolerant toxicity have reported different results both in classical methods (Suhartini *et al.,* 1996; Suhaimi, 1992; Abifarin, 1986) and molecular quantitative trait loci (QTL) methods (Dufey *et al.,* 2009, 2012; Shimizu 2009; Wan *et al.,* 2003, 2004, Wu *et al.,* 1997, 1998). This varying information is probably because of the difference in using of prediction method for genetics parameters or the method on quantifying evaluation system on visual scoring iron toxicity symptom. Most common visualization scoring system is developed by IRRI (Standard Evaluation System for Rice, http://www.knowledgebank. IRRI.org/ses/SES.htm). This system has been useful to evaluate tolerance and is used in the breeding of tolerant rice to iron toxicity. However, this scoring has an inherent weakness in its statistical applicability for instance, there is no theoretical basis to consider between scores 1 and 2, scores 2 and 3, and etc. Therefore, genetic analysis cannot be correctly applied to the bronzing score. Another issue that should be addressed that scoring scales cannot be used for genetics analysis because quantitative data is necessary. Hence it is important to convert the scoring scale to quantitative data.

In plant analysis, tolerance level is determined by appearance of color variations of leaves and or others plant's part due to different genotypes and or injuries because of exposure to particular stress (Fiorani and Schurr, 2013). The color variation and intensity of color can be measured by computer-aid method, so called the RGB color model in which red, green, and blue (Leon *et al.,* 2006). Various experiment has used RGB analysis in the method of plant analysis to determine response of plant to the treatment, namely diagnostic harvesting time of rice (Iwaya and Yamamoto, 2005), prediction of chlorophyll content (Yadav *et al.,* 2010), and salinity stress analysis in rice seedling (Hairmansis *et al.,* 2014).

The RGB method is able to measure the colors' change and its intensity from green (tolerant) to brownish-red (sensitive) in leaf bronzing symptom because of iron toxicity. Here we studied possibility of the use of RGB method for quantifying bronzing score and its application in genetics analysis. We described the results of a quantitative genetic study in crosses of Pokkali, an iron-tolerant variety with robust development of seedling type (Engel *et al.,* 2012), as well as tolerant to salinity (Gregorio *et al.,* 2002) and Mahsuri, an iron-toxicity tolerant varieties which is well-known in Indonesia (Suhartini, 2004) using generation mean analysis approach (Mather and Jinks 1982).

MATERIALS AND METHODS

Plant material

The parental screening and validation of RGB method for iron toxicity tolerance used 23 genotypes of known degree of tolerance of iron toxicity based on previous study of field studies and different of origin (Table 1). Based on this parental screening we selected rice varieties to develop six generation populations. The rice varieties Pokkali and *Mashuri* were used as tolerant parents to iron toxicity, while Inpara 5 as sensitive parent. These varieties were crossed in a resulting of populations each composed of six generations per cross. The F_1 between the 2 varieties was produced in the dry season 2013.

Table 1. Origin and designation of various rice genotypes used in this study.

No	Genotype	Origin	Tolerance to iron toxicity
1.	IR64	lowland rice, IRRI[a]	Sensitive
2.	IPB107F-5-1-1	Swampy rice, BAU[b]	Unknown[d]
3.	Inpara5	lowland rice, IRRI	Sensitive[f,g]
4.	Fatmawati	lowland rice, ICRR[c]	Unknown[g]
5.	Batu Tegi	Upland rice, ICRR	Unknown[g]
6.	IPB Kapuas 7R	Swampy rice, BAU	Tolerant[e]
7.	IPB Batola 6R	Swampy rice, BAU	Tolerant[e]
8.	A. Tenggulang	Swampy rice, ICRR	Tolerant[g]
9.	Indragiri	Swampy rice, ICRR	Tolerant[g,hj]
10.	B13100-2-MR-2	Swampy rice, ICRR	Tolerant[i]
11.	IPB Batola 5R	Swampy rice, BAU	Tolerant[e]
12.	Limboto	Upland rice, ICRR	Unknown[g]
13.	IPB1 R	Swampy rice, BAU	Moderate[g]
14.	Dadahup	Swampy rice, BAU	Tolerant[e]
15.	Mahsuri	Swampy rice, Local	Tolerant[k]
16.	Mesir	Lowland rice, Local	Unknown[n]
17.	Inpara 2	Swampy rice, ICRR	Tolerant[g]
18.	Margasari	Swampy rice, ICRR	Tolerant[g]
19.	B13144-1-MR-2	Swampy rice, ICRR	Tolerant[k]
20.	Cilamaya	Lowland rice, ICRR	Tolerant[g]
21.	Kapuas	Swampy rice, Local	Unknown[n]
22.	Pokkali	Lowland rice, India	Tolerant[o]
23.	Awan Kuning	Swampy rice, Local	Tolerant

[a] International Rice Research Institute; [b] Bogor Agricultural University; [c] Indonesia Center for Rice Research; [d] Recommended sensitive check for iron toxicity in rice (Suhartini and Makarim, 2009); [e] Released rice variety in Indonesia by IPB (http://dri.ipb.ac.id/PDF_file/Buku_varietas_3%Feb%202014.pdf); [f] NILs of IR64 carrying Sub1 previously IRRI designation was IR84194-139 (Septiningsih *et al.*, 2015); [g] Released rice variety in Indonesia by Ministry of Agriculture (http://bbpadi.litbang.pertanian.go.id/index.php/publikasi/buku/content/item/150-deskripsi-varietas-padi-2013); [h] Tested on iron toxicity tolerance in South Sumatra (Harahap *et al.*, 2014); [i] Top high yield line in advance yield trial in Sumatera (Nugraha *et al.*, 2012); [j] Tolerant parent from genetic study on Iron Toxicity rice (Suhartini, 2004); [k] Most tolerant identified in field and greenhouse screening for iron toxicity (Suhartini and Makarim, 2009); [l] Local variety from Kalimantan and Sumatera (Suhartini, 2004); [m] Excluder types tolerant to iron toxicity (Engel *et al.*, 2012), tolerant to salinity (Gregorio *et al.*, 2002)

Several F_1 plants were grown in the wet season 2013 to produce selfing seed, F_2 and the first backcross generations, BC_1P_1 and BC_2P_2. For each cross, individual plants evaluated varied by generation based on the expectation of genetic segregation; therefore, more individuals were evaluated in the F_2, and BC generation than in the parents and F_1 (Table 2).

Plant growth conditions

The experiment was done in greenhouse facility of Indonesian Center for Rice Research, Bogor Indonesia in March to May, 2014. The seeds of six generations were surface-sterilized with 5% $NaClO_3$ sodium hypochlorite for 15 min and thoroughly washed with tap water and soaked in water at 30°C then placed in the dark for 3 days until germination. Plants were grown in a greenhouse with daily average temperature of 29.5°C and relative humidity of 85%. The rice seedlings were transplanted to drilled Styrofoam trays at the desired planting density (100 holes per 10 L) and placed to Yoshida solution (Yoshida *et al.*, 1976) with the following composition: NH_4NO_3 1.42 mmol L^{-1}, $K_2PO4.2H2O$ 0.05 mmol.L.1, K_2SO4 0.5 mmol.L^{-1}, $CaCl_2$.$2H_2O$ 1 mmol.L^{-1},$MgSO_4.7H_2O$ 1 mmol.L.1, $MnCl_2.4H_2O$ 9 µmol.L^{-1}, $(NH_4)6Mo_7O_{24}.4H_2O$ 0.07 µmol.L^{-1}, H_3BO_3 18.5 µmol L^{-1}, $CuSO_4.5H2O$ 0.16 µmol L^{-1}, $FeSO_4$ 36 µmol L^{-1}, dan $ZnSO_4.7H2O$ 0.15 µmol L^{-1}. After pre-treatment, the 2week-old rice seedlings were transferred to high iron-treated environment which were given by adding $FeSO_4$ as much as 400 mgL^{-1}. We prepared

Table 2. Generations and number of plants evaluated per generation (n) in 2 generation means experiments planted in greenhouse experimental station, Bogor, 2014.

Generation	Cross I	n Cross I	Cross II	n Cross II
P_1	Inpara 5	17	Inpara 5	17
P_2	Mahsuri	18	Pokkali	20
F_1	P_1 x P_2	19	P_1 x P_2	20
F_2	F1 self	187	F1 self	195
BC_1P_1	F_1 x Inpara 5	50	F_1 x Inpara 5	38
BC_2P_2	F_1 x Mahsuri	54	F_1 x Pokkali	40

solution to anaerobic conditions by adding agar solution to reduce the precipitation of Fe^{2+} to Fe^{3+} (Colmer, 2003). The solution was subsequently added by 20 g agar which is dissolved in 1 L of boiling deionized water. Agar solution was allowed to cool to approximately 60°C and then mixed to the solution in the trays (the final agar concentration was 0.2% [w/v]). The trays were filled with 10 L with deionized water and the pH of the medium was adjusted to pH 5.6–5.7.

Phenotyping

The extent of leaf bronzing was scored at 10-d after treatment. The leaf bronzing scores were determined using scoring scale, 1 (no bronzing symptom on the leaf) to 7 (the whole leaves were bronzing), developed by Shimizu *et al.* (2005) at ten days after Fe^{2+} treatment. The samples were uprooted from the media and washed using tap water for determining standardized score index of bronzing. The entire part of plant sample including shoot and root was photographed using a pocket camera (Nikon coolpix S6400). The pictures were taken in a dark room and the samples were placed on black sheet, to avoid disturbing light from others sources. The camera was placed on a tripod at a distance as 75 cm from the samples. The digital images then analyzed using pixels in 3 primary colors: red, green, and blue (RGB) components by using Adobe Photoshop CS3© (Adobe Systems Inc., USA). To reduce the noise, the background of the pictures was changed to white using magic wand tool and mean values of each of RGB component per plant shoot were used as digital-converted data. We did not measure RGB component on root since it had no different among genotypes. We chose R/G for the quantification of bronzing as the leaf discoloration progresses from green to red.

The samples also were measured for other parameters related to the iron toxicity tolerance. Shoot length was measured from basal to highest tips of the leaf. The root length was measured from root basal to the tips of the root; the shoot fresh weight was measured by weighing the sample after wrapped gently with paper towel. We did not measure dry weight on generation mean experiment because the selected samples would be grown to produce seeds to regenerating the population.

Shoot iron content analyses were done using acid digestion following the method described by Indonesian Soil Research Institute (http://balittanah.litbang.deptan.go.id). In brief the samples were oven dried at 70^{o} C for 3 days. The oven-dried shoot samples then measured for shoot dry weight. The samples subsequently were ground and weighed 0.5 g into digestion tube. The sample were digested using 5 mL concentrate acid (HNO_3:$HClO_4$ = 3:1). On the following days, sample was heated on digestion block at 120^{o}C for 24 hours. After the tube had cooled, the digest was transferred to 25 mL flask with deionized water. Iron plaque and shoot concentration were measured by atomic absorption spectrophotometry.

Data analysis

A joint-scale test was performed using chi-square goodness of fit with 3 degrees of freedom as described by Cavalli (1952). When the 3-parameters individual-scaling model did not show conformity of additive dominance (i.e. with values different from zero), six-parameter

scaling model (m = ½P_1+½P_2 + 4F_2 – 2BC_1P_1 – 2BC_1P_2; d = ½P_1 – ½/P_2; h = 6BC_1P_1 + 6BC_1P_2 – 8F_2 – F_1 – 1½P_1 – 1½P_2; i = 2 BC_1P_1 + 2 BC_1P_2 – 4F_2; j = 2 BC_1P_1 – P_1 – 2 BC_1P_2+P_2; l = P_1 + P_2 + 2F_1 + 4F_2 – 4 BC_1P_1 – 4 BC_1P_2) was performed to include the contribution of a digenic epistasis (nonallelic interaction). The test provides estimates for 3 parameters [mid-parent (m)], additive effect (d), and dominance effect (h). It also provides estimates for 3 epistasis parameters; additive x additive (i); additive x dominance (j) and dominance x dominance (l). A significant level ($P \leq 0.05$) was used to compare all components. The 3- and 6-parameter models were developed as described by Mather and Jinks (1982).

Broad-sense heritability was estimated using the method described by (Fehr 1987) as:

$$h^2_b = \frac{\sigma^2_g}{(\sigma^2_{g+}\sigma^2_e)}$$

The estimate of genetic variance (σ^2_g) is equal to the variance of F_2 generation (σ^2 F_2) minus the environmental variance (σ^2_e). In this formula, σ^2_e = [nP_1 σ^2P_2 + nP_2 σ^2_{P2} + nF_1 σ^2F_1]/Ne; where nP_1, nP_2 and nF_1 refer to the number of plants of sensitive parents (P_1), resistant parents (P_2) and F_1 generations, respectively. The term Ne refers to effective population size, where Ne = nP_1 + nP_2 + n_{F1}, i.e., number of P_1, P_2 and F_1, respectively. The method used to estimate narrow-sense heritability was adapted from (Fehr, 1987):

$$h^2_n = \frac{2(\sigma^2 F_2) - (\sigma^2 BC_1 + \sigma^2 BC_2)}{\sigma^2 F_2,}$$

Where σ^2 F_2 is the variance among F_2 individuals, σ^2BC_1 and σ^2BC_2 are the variances of BCP_1 and BCP_2 generations, respectively. Correlation between related traits was performed using simple Pearson correlation.

The mean and correlation of all observed traits were analyzed using SAS/STAT® version 9.1. (SAS Institute, 2004). The SAS listing program for the scaling test of 3 and 6 parameters and heritability analysis were developed by Gusti N. Adi-wibawa, and may be obtained by contacting the corresponding author.

RESULTS

Parental Screening and validation of RGB methods for iron toxicity tolerant

There was variation of R/G and LBS among genotypes (Figure 1). The green color intensity of tolerant variety was higher compared to sensitive variety and vice versa for the red color intensity (Figure 2). All genotypes that categorized as sensitive or tolerant with LBS also showed the same tendency as with the R/G methods. Only few genotypes deviated in categorizing as moderate using both LBS and R/G. For example, IPB Kapuas 7R and Batu Tegi were categorized as sensitive using LBS but moderate using R/G. This probably is because of narrow differentiation and weakness of visual observation between tolerance and moderate, and vice versa. However, overall there was strong correlation between LBS and R/G using simple correlation, r = 0.854** (Table 3). The R/G ranged from 1.17, the most sensitive variety, IPB107-5-1-1, to 0.95, the most tolerant variety, Siam Saba. Based on this result, we selected the sensitive parent, Inpara 5, and the tolerant parent Mahsuri and Pokkali for further study.

We also confirmed that R/G method was correlated with shoot iron content but it was not correlated with shoot dry weight (Table 3). This because there was variation on shoot dry weight in relation to tolerance to iron toxicity, for example the iron-tolerant variety, Pokkali is the robust seedling growth genotypes while other tolerant variety like Mahsuri and *Siam Saba* are iron toxic-tolerant varieties with low seedling dry weight-type genotypes. This differentiation also used as consideration in choosing parental for this genetics study.

Table 3. Simple correlation of RGB and related traits to tolerance of rice seedling under high level of iron (N = 23).

	R/G	LBS	Shoot dry weight	Shoot iron content
R/G	1	-0.850**	0.222	-0.460*
LBS		1	-0.376	0.216
Shoot dry weight			1	-0.067
Shoot iron content			-	1

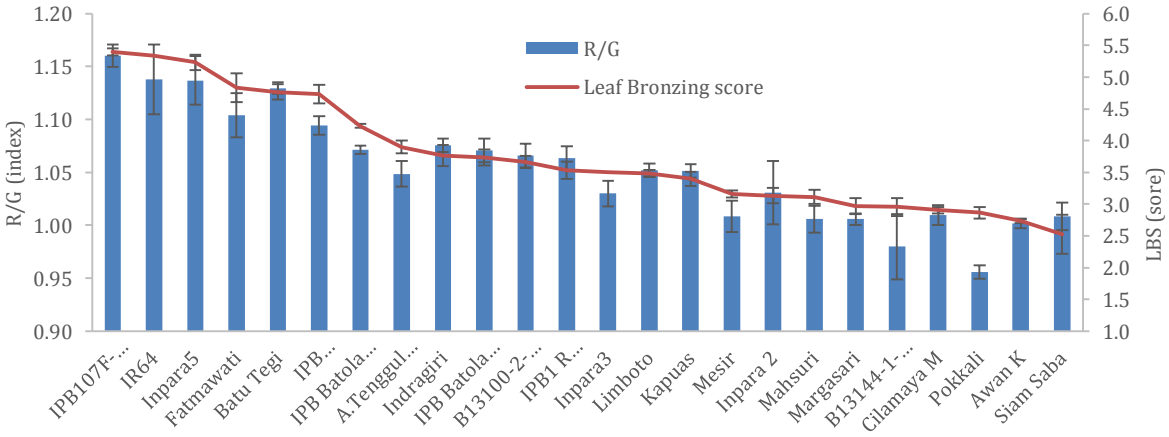

Figure 1. R/G index and LBS of seedling 23 rice genotypes under high iron concentration of 400 mg.L^{-1}.

Figure 2. Appearance and their R/G index of rice seedling processed by Adobe Photoshop CS3©.

Generation mean and gene effect

The Mahsuri and Pokkali (tolerant parents) means were greater than the Inpara 5 (sensitive parent) mean for most of the traits, except for shoot length of Inpara 5 and Mahsuri was no significant different (Table 4). Overall the F_1 mean of all the traits were greater than both of the parents in the cross of Inpara 5 x Mahsuri. While the F_1 mean of the cross of Inpara 5 x Pokkali in most of traits were in the range between two-parent mean. The F_2 means for most traits were in the range of the parents mean as well. Backcross means of the F_1 to the superior parent (Mahsuri and Pokkali) showed higher values for most traits compared with back

Table 4. Mean and deviation of population P_1,P_2, F_1, F_2, BC_1P_1, dan BC_1P_2 of rice seedling of Inpara 5 x Mahsuri dan Inpara 5 x Pokkali under high Fe concentration (400 mg.L^{-1}) for 10 days.

Population	R/G (index)		Shoot length (cm)		Root length (cm)		Fresh weight (g)	
	Inpara 5 x Mahsuri	Inpara 5 x Pokkali	Inpara 5 x Mahsuri	Inpara 5 x Pokkali	Inpara 5 x Mahsuri	Inpara 5 x Pokkali	Inpara 5 x Mahsuri	Inpara 5 x Pokkali
P_1	1.10±0.02	1.09±0.04	23.15±2.45	23.29±1.88	5.80±0.83	5.95±0.83	0.31±0.02	0.33±0.03
P_2	0.91±0.05	0.89±0.03	24.10±2.57	41.82±2.70	10.10±1.25	17.11±1.28	0.45±0.03	0.83±0.08
F_1	0.90±0.04	0.91±0.03	28.87±2.75	39.21±2.70	10.21±1.03	10.64±1.20	0.46±0.05	0.48±0.07
F_2	0.94±0.06	0.98±0.09	21.94±4.41	30.65±6.36	8.47±2.27	8.76±2.41	0.37±0.09	0.40±0.15
BC_1P_1	1.02±0.04	1.03±0.08	19.57±4.17	26.68±4.88	6.26±2.13	7.30±1.78	0.29±0.09	0.31±0.11
BC_1P_2	0.93±0.05	0.91±0.07	21.25±4.22	33.00±5.26	7.33±1.79	8.97±2.73	0.33±0.09	0.50±0.11

Table 5. Mean and deviation of population P_1,P_2, F_1, F_2, BC_1P_1, and BC_1P_2 of rice seedling from cross of Inpara 5 x Mahsuri and Inpara 5 x Pokkali under high Fe concentration (400 mg.L^{-1}) for 10 days.

Parameter	R/G (index)		Shoot length (cm)		Root length (cm)		Fresh weight (g)	
	Inpara 5 x Mahsuri	Inpara 5 x Pokkali	Inpara 5 x Mahsuri	Inpara 5 x Pokkali	Inpara 5 x Mahsuri	Inpara 5 x Pokkali	Inpara 5 x Mahsuri	Inpara 5 x Pokkali
Three parameters[a]								
m	1.00+0.006***	0.99+0.04***	21.52+ 0.36***	31.10+0.34***	7.43+ 0.16***	10.91+ 0.17***	0.37+ 0.04***	0.56+0.01***
d	-0.09+0.004***	0.11+0.04***	0.43+0.36^{ns}	8.27+0.35***	1.90+ 0.16***	4.97+0.17***	0.07+0.04***	0.23+0.01***
h	-0.09+0.011***	-0.06+0.0***	2.13+ 0.70***	3.28+0.35***	1.97+0.29***	-3.61+0.34***	0.02+0.01^{ns}	-0.25+0.02***
X^2	23.0(p<0.001)	19.7(p<0.001)	161.5(p<0.001)	140(p<0.001)	75.6(p<0.001)	52.6(p<0.001)	54.4(p<0.001)	36.5(p<0.001)
Best fit[b]								
m	0.86+0.03***	1.06+0.02***	32.09+1.91***	32.8+0.37***	14.86+1.8***	11.53+0.19***	0.63+0.05***	0.58+0.01***
d	-0.09+0.06***	0.10+0.06***	0.32+0.36ns	9.00+0.37***	2.02+0.15***	5.58+0.19***	0.07+0.00***	0.25+0.01***
h	0.31+0.07**	-0.15+0.02***	-37.41+5.25***	-15.96+1.77***	-20.92+3.13***	-8.74+0.82***	-0.82+0.14***	-0.50+0.05***
i	0.14+0.03**	-0.08+0.02***	-8.20+1.86***	-	-6.96+1.6***	-	-0.26+0.05***	-
j	-	0.03+0.03ns	-	-5.19+2.44***	-	-6.99+1.05***	-	-0.14+0.06***
l	-0.27+0.06***	-	34.22+3.56***	22.45+1.92***	16.26+2.04***	5.85+0.92***	0.63+0.09***	0.30+0.05***
X^2	3.24(0.57)	0.05(0.95)	0.27(0.60)	1.40(0.20)	5.7(0.02)	4.44(0.035)	2.37(0.123)	0.10(0.75)
Epistasis[c]	duplicate epistasis I	Partial dominance D	duplicate epistasis D	duplicate epistasis D	duplicate epistasis D	duplicate epistasis D	duplicate epistasis D	duplicate epistasis D

[a] Mean (m), additive (d), dominance (h), additive x additive (i), additive x dominance (j), dominance x dominance (l)
[b] X^2 test with 1 df for the 5 parameters model *, **, and *** significantly from zero at 0.05, 0.01, and 0,001 probability level according to student t test
[c] Type of epistasis. *I*, increasing effect to favorable allele, *D*, Decreasing effect to favorable allele

cross means to the inferior parent (Inpara 5).

The simple additive-dominance model using 3 parameters of generation means showed that the 3 terms (m, d and h) to be significant for all traits, except the dominant component of R/G in the all crosses, the additive component of shoot length in the cross of Inpara 5 x Mahsuri and the dominant component of fresh weight in the cross of Inpara 5 x Mahsuri cross (Table 5). However, χ^2 test showed that the simple additive-dominance model was not fitted in all traits. Hence, this indicated the presence of non-allelic gene interactions or epistasis on the scale of measurement used.

Based on the best fit parameter model, the additive (d) effects were positive while the dominance (h) effect were negative in all traits except for R/G index in the cross of Inpara 5 x Mahsuri (Table 5). This indicated that alleles which decreasing in the particular traits were more important in the gene effect. A five parameter model involving additive x additive (i), dominance x dominance (j) epistasis, in addition to additive (d) and dominance (h) components were the best fit model to explain the variances among the generations for the Inpara 5 x Mahsuri cross in all traits. Meanwhile, for most of the traits in the Inpara 5 x Pokkali cross, a five parameter model involving additive x dominance (j) and dominance x dominance (l) epistasis in addition to additive (d) and dominance (h) components were necessary to describe the variances among the generations. However, the net dominance (h) and dominance x dominance (l) effects of those the 2 crosses showed different directions, indicating that the type of epistasis was duplicate epistasis between dominant with decreasing effect to favorable allele (Kearsey and Pooni, 1996). In the exception for R/G in Inpara 5 x Pokkali cross, five parameters additive x additive (i), additive x dominance (j) were to be determiner of variances. The net of additive (d) and dominance (a) effects were similar, indicating complete dominance association alleles within the parent.

Heritability

Broad-sense heritability (h^2_b) and narrow-sense heritability (h^2_n) for components iron toxicity tolerance are shown in Table 6. Heritability estimates ranged moderately from 0.61 to 0.88 for broad-sense heritability and 0.02–0.65 for narrow sense in those 2 crosses. The highest heritability was recorded for shoot length in the cross of Inpara 5 x Pokkali (h^2_b = 0.86 and h^2_n = 0.65, for broad sense and narrow sense heritability, respectively). R/G index had moderate broad sense heritability in all crosses (0.70 and 0.85), and low for the narrow-sense heritability (0.39 for Inpara 5 x Mahsuri cross and 0.55 for Inpara 5x Pokkali).

Correlation of F_2 population

R/G index showed strong correlation with most traits, except for shoot length of Inpara 5 x Mahsuri cross (Table 7). Low correlation of shoot length of Inpara 5 x Mahsuri also was found with other traits, but not for root length, indicating low variation of root more important than the shoot in relation to iron toxicity tolerant in this population. Although there was no strong relationship within shoot and root length in Inpara 5 x Pokkali cross, however this has strong relationship with LBS and R/G.

Table 6. Heritabilities of F_2 population from cross of Inpara 5 x Mahsuri and Inpara 5 x Pokkali under high Fe concentration (400 mg.L^{-1}) for 10 days.

Heritability	R/G (indexes)		Shoot length (cm)		Root length (cm)		Fresh weight (g)	
	Inpara 5 x Mahsuri	Inpara 5 x Pokkali	Inpara 5 x Mahsuri	Inpara 5 x Pokkali	Inpara 5 x Mahsuri	Inpara 5 x Pokkali	Inpara 5 x Mahsuri	Inpara 5 x Pokkali
h^2_b	0.70	0.85	0.73	0.86	0.81	0.80	0.84	0.81
h^2_n	0.39	0.55	0.02	0.65	0.43	0.16	0.10	0.22

h^2_b, broad sense heritability, h^2_n, narrow sense heritability

Table 7. Simple correlation of F_2 population of some traits related tolerance to iron toxicity in rice seedling (N = 200).

Traits	LBS	R/G	Shoot length	Root length	Fresh weight
LBS	1	-0.784***	0.045	-0.417*	-0.190
R/G	-0.964***	1	0.064	0.496**	0.575**
Shoot length	-0.359*	0.481**	1	0.179	0.271
Root length	-0.062	0.627***	0.058	1	0.407*
Fresh weight	-0.514**	0.634***	0.400*	0.397*	1

The values above the diagonals are simple correlation coefficient of Inpara 5 x Mahsuri cross and below the diagonal is simple correlation coefficient Inpara 5 x Pokkali cross.

DISCUSSION

Breeding for iron toxicity tolerance has been undertaken based on visual appearance of leaf color change to yellowish reddish or leaf bronzing as reaction of rice plant to excess iron in the field or in the greenhouse. This system certainly causes a personal error, or inherently requires skillful eye in selection practices. An alternative to visual rating used a portable chlorophyll meter (SPAD) has been conducted in field research (Audebert and Sahrawat, 2000). However, these measurements may not completely explain spread of leaf discoloration on leaf bronzing because of limited measurement area of 2 × 3 mm^2 and this machine was only effective in gradation of green color but did not match to reddish or brownish in bronzing leaf. Improvement of visual rating was made by Shimizu (2009) using digital image by capturing leaf blade images using scanning printing machine. This method used a detached leaf from the shoot that would destruct the sample. In this study, we utilized pocket camera that would be able to measure not only individual the leaf but also image of the whole plant. However, we did not measure color intensity in the root because of over lapping of red-brownish color within root system. This quantification using R/G index can easily be extended to other visually scored indexes for various abiotic and biotic stresses, and can be used in a statistically because providing continuous data, which required in quantitative genetics analysis.

In this study, we identified variation of 23 genotypes in reaction to iron stress (Figure 1). Among screened genotypes under iron toxic culture media solution we chose Mahsuri and Pokkali as tolerant parents. Similar ranking was reported for iron toxic tolerant rice for Mahsuri (Silitonga, 2004) and this variety was commonly used as donor parent for iron toxicity in rice improvement in Indonesia (Suhartini, 2004). Meanwhile Pokkali was also reported as tolerant parent which having characteristics of excluder type and robust seedling growth (Engel *et al.*, 2012). The R/G value and LBS of those 2 varieties was similar, but the distinct characteristic of those varieties was in shoot dry matter where Pokkali was the highest among tested genotypes (data not presented). We used Inpara 5 as sensitive parent. There was no report previously regarding sensitivity of this variety to iron toxicity. However, this variety was the near-isogenic line of IR64 that introduced a Sub1 locus on top of chromosome 9 (Septiningsih *et al.*, 2009) and has been released as rice variety in Indonesia (Septiningsih *et al.*, 2015). Our findings also showed that Inpara 5 and IR64 had both of LBS and R/G similar in the reaction to iron toxicity media solution. This means that Sub1 had no effect on tolerance to iron toxicity.

It has been reported that most genetic studies of iron toxicity rice tolerant reveal to quantitative inheritance involving many genes, although Abifarin (1986) reported a single recessive gene governed in variety *Gissi 27*. Using classical genetic study, Suhartini *et al.*, (1996) used diallel analysis and Suhaimi (1992) used generation mean analysis had reported complex genetics control involving additive, dominance and allelic interaction. Genetics studies using molecular marker under various environmental conditions and using different segregating populations (Dufey *et al.*, 2009, 2012; Shimizu 2009; Wan *et al.*, 2003, 2004; Wu *et al.*, 1997, 1998) also reported small effect

of QTLs on iron toxicity tolerant, indicating complex genetic effect playing important role in the inheritance of this traits. Nevertheless, the challenges of confident genomic localization remain enormous, and with several hundred genes involved in iron toxicity tolerant traits (Dufey *et al.*, 2014).

The findings of this study indicated most of the traits were affected by additive (d) effect which was positive while the dominance (h) effect was negative. This indicated that among alleles, the decreasing allele were more important than those which increasing in the particular traits. This report was probably similar with role of recessive gene on iron toxicity tolerance reported by Abifirin (1986). Since that report used discontinuous scoring index resulting in rough discrimination of tolerance level among population. However, this present study used a quantification to measure the level of tolerance resulting continuous data that can facilitate the analysis using generation means (Mather and Jinks, 1982).

These results have implication for breeding and selection of improved iron toxicity tolerance. For the traits showing duplicate epistasis, the procedure of selection should be modified to exploit their inter-allelic interaction. This includes selection in later generations and maintenance of large populations prior to selection to provide maximum opportunity for advantageous combination of genes to occur (Witcombe and Virk, 2001). Early generation selection would be less effective. Maintenance of large populations could be particularly necessary when combined with other important traits using other genotypes. Those are included in breeding programs because in the adapted and other traits in the crosses will be segregating as a geometric function of number of segregating loci. Further, in studies involving adapted and novel crosses, it is advantageous to backcross one or more times with recurrent parent before selection to enhance the probability of obtaining superior lines (Dudley, 1982). In practice it is possible to investigate the larger population for leaf bronzing screening using digital analysis of R/G method. This method will also help molecular breeding efforts for the identification of robust QTLs or markers using a quick and repeatable phenotyping through high-throughput screening of Fe-toxicity-tolerant genotypes under controlled conditions.

We conclude that by using R/G index, the leaf bronzing score of rice iron toxicity can be quantified, and applied to statistically-analyzed genetics study. The results of this study indicated that tolerance to high iron appears to be quantitatively inherited and the complexity in the crosses were reported here. Iron tolerance in rice is a heritable character which could be successfully selected in late generation. Breeding methods that make a better use of additive and epistasis variance should be used, such as recurrent selection.

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