



Stability and $G \times E$ analysis of zinc-biofortified rice genotypes evaluated in diverse environments

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Abstract Rice is the major staple food and source of energy in Asia, particularly in the Philippines. Most of the popular, high-yielding rice varieties lack sufficient amount of micronutrients to meet daily human requirements. Micronutrient deficiencies are a major health burden globally and can cause severe health problems. In the Philippines, nearly one-third of the population is zinc (Zn) deficient. Breeding healthier rice varieties is one of the major sustainable interventions to tackle micronutrient deficiencies. However, genotype and environment interactions ($G \times E$) are a major obstacle in breeding for high-Zn rice varieties, thus an understanding of $G \times E$ interactions and identifying stable genotypes through multi-location evaluation will help in identifying potential lines for

varietal release. We evaluated eight high-Zn rice breeding lines along with checks in several locations across the Philippines during 2014 wet season (WS), 2015 dry season (DS) and 2015WS, and 2016DS. Individual and combined analysis of variance revealed significant genotypic effects and $G \times E$ interactions for all the traits studied. Significant positive correlations between grain iron (Fe) and Zn, days to maturity, and plant height were observed, whereas grain yield and Zn were negatively correlated. Stability analysis using Kang's stability index, additive main effects and multiplicative interaction, and genotype main effects plus $G \times E$ (GGE) consistently identified IR10M300 as the most stable genotype across seasons in terms of yield and grain Zn. Over all IR10M300 has a yield of 4690.3 kg ha⁻¹ and grain Zn of 18.3 ppm. It outperformed two rice variety checks (PSBRc82 and MS13) for grain Zn with comparable yield with the yield check (PSBRc82) and was successfully released as the

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first High-Zinc Rice 1 (NSICRc 460) in the Philippines.

Keywords Rice · Biofortification · Zn · $G \times E$ · Stability · AMMI · GGE · NSICRc460 · High-Zinc Rice 1

Abbreviations

E or ENV	Environment
G or GEN	Genotype
HT	Plant height
IPCA	Interaction principal components axes
IRRI	International Rice Research Institute
MET	Multi-environment trial
MT	Days to maturity
NCT	National Cooperative Testing
PhilRice	Philippines Rice Research Institute
PC	Principal component
SD	Standard deviation
TN	Tiller count
YLD	Grain yield

Introduction

Breeding rice varieties with improved health and nutrition value is one of the priority areas of rice research. Modern agriculture has been successful in addressing food security to a large extent, while nutritional security remains a major challenge in the developing world (Bouis et al. 2013). Micronutrient deficiency malnutrition affects more than two billion people worldwide. In the Philippines, one-third of the population is at risk of Zn deficiency (Wessells and Brown 2012; FNRIDOST 2015). Zn is a major cofactor for several vital enzymes involved in human metabolism; hence, it is highly essential for the normal growth and development of human beings (Keith et al. 2006; Roohani et al. 2013; Sadeghzadeh 2013). Zn deficiency causes diarrhea, growth retardation, loss of appetite, impaired immune function, cognitive defects, skin rashes, etc., especially in children and pregnant and lactating women, and these problems are highly prevalent in rice-consuming Asian populations (IMFNB 2001; Hotz and Brown 2004; Prasad 2004, Wang and Busbey 2005).

Rice is the major staple food for Asians and it is eaten in significant quantities on a daily basis.

However, most of the rice consumed is milled and has lesser amounts of grain Zn (Kennedy et al. 2002; Sharma et al. 2013). The development of rice varieties with higher levels of bioavailable Zn has been suggested to be a sustainable, targeted, food-based, and cost-effective approach for alleviating Zn-deficiency malnutrition (Bouis and Welch 2010). In breeding for commercially successful high-Zn rice, high yield potential is equally important, but these two traits have complex genetic inheritance and are highly influenced by environmental factors. Several environmental factors such as soil structure and texture, soil pH, organic matter content, soil nutritional status, soil flora and fauna, soil redox potential, sulfur and bicarbonate content, irrigation, agronomic management practices, etc., influence soil Zn availability, root uptake, translocation, and loading of Zn into rice grains and also the yield performance of genotypes (Graham et al. 1999; Hacısalihoglu and Kochian 2003; White and Broadely 2011; Impa and Johnson-Beebout 2012; Rubianes et al. 2017).

Genotype by environment interactions ($G \times E$) are a major obstacle for developing and releasing high-Zn rice varieties. A stable-performing line across environments and seasons is needed to reap the benefits of genetic gain for grain Zn, yield, and yield-related traits in farmers' fields. A clear understanding of $G \times E$ interactions is essential in identifying stable genotypes for recommending a varietal release. $G \times E$ interactions vary significantly with the genotype, test environment, and genotype response to the environment (Fox et al. 1997; Malosetti et al. 2013). Breeding programs include Multi-Environment Trials (MET) as part of the final stages in evaluating advanced breeding lines to assess the stability and adaptability of potential lines to be released as varieties.

Breeding activities for the development of high Zn rice varieties received a major impetus with the launch of the HarvestPlus-led global biofortification program in 2003. Through conventional breeding, high grain Zn traits have been incorporated into elite, high-yielding germplasm at International Rice Research Institute (IRRI) (Swamy et al. 2016). High Zn rice varieties have been released for cultivation in Bangladesh and India. Advanced high Zn breeding lines are under different stages of evaluation in Indonesia, Myanmar, Cambodia and Vietnam. There is huge demand and need for high Zn rice varieties in these countries with bigger scope and opportunities in

developing new varieties targeted to each of these countries. However, understanding the $G \times E$ and identifying stable high Zn lines is essential for the successful release and dissemination of high Zn rice lines in each of the target countries. The NCT program of the Rice Varietal Improvement Group (RVIG) in the Philippines assessed the performance of eight high Zn rice lines at representative rice growing regions of the Philippines.

Advanced statistical tools are essential to dissect $G \times E$ for various complex traits. Analysis of variance can detect $G \times E$ interactions and stability models determine stable genotypes. Different univariate and multivariate statistical models are being routinely used for stability analysis and in identifying stable genotypes for different traits. Univariate models include regression analysis (Eberhart and Russell 1966), coefficient of variation (Francis and Kennenbergh 1978), mean sum of squares (Lin and Binns 1988), Shukla's (1972) stability model, and yield stability index (YSi) proposed by Kang (1993). The YSi statistic uses the mean, while Shukla's stability model uses variance in identifying stable genotypes for a particular trait. Multivariate models such as additive main effects and multiplicative interaction (AMMI) model and the genotype main effects plus $G \times E$ (GGE) model are the most widely used statistical models for the analysis of data from multi-environment testing of rice genotypes to understand $G \times E$ interactions (Gauch 2006).

The AMMI model makes use of a two-way analysis of variance (ANOVA) and principal component analysis (PCA) to compute additive main effects and interaction effects, respectively. The GGE biplot model uses one-way ANOVA for estimating genotypic effects and $G \times E$ interactions (Yan et al. 2000). In AMMI, $G \times E$ effects is plotted in a biplot, whereas, in GGE, the genotype and $G \times E$ effects are presented using environment-centered PCA. Both of these models have their own merits, yet GGE has an advantage in presenting the what-won-where pattern, which can help in identifying mega-environments and $G \times E$ crossover (Gauch and Zobel 1997).

Our present study covered the results of National Cooperative Testing (NCT) for high Zn rice in the Philippines. The main objectives of our study were to evaluate the performance of high-Zn rice genotypes across different environments in the Philippines, to

assess the $G \times E$ interactions for agronomic, yield, and grain Zn traits, and to identify the most stable genotypes for grain yield and Zn.

Materials and methods

This study used four season's data (2014WS, 2015DS, 2015WS, and 2016DS) from the NCT for special purpose rice in the Philippines.

Experimental sites

The field trials were conducted in different agro-ecological zones in the Philippines. Details on soil and climate characteristics for the test locations are presented in Table S1. The genotypes were tested in nine different environments (E1 to E9) during the WS (June to December) but only eight environments (E1 to E8) during the DS (January to May). These testing environments well represented the major rice growing areas of the Philippines and have been used for varietal identification and release by the Philippine Rice Research Institute (PhilRice). The soil type was clay loam to loamy sandy in most of the locations and the annual rainfall in these locations was more than 1600 mm. The soil pH was slightly acidic in most of the location except in Batac. Soil pH ranged from 5.0 to 7.8.

Experimental materials

The high Zn breeding materials developed at IRRI and PhilRice were used as test materials for this experiment. In total ten genotypes including eight high Zn rice lines, yield check and micronutrient checks were evaluated during 2014WS, 2015DS, 2015WS and 2016DS. MS13 (G7), the first biofortified variety released under the Special Purpose rice category was used as a micronutrient check and a popular high yielding rice variety with good grain quality in the Philippines PSBRc82 (G10) was used as a yield check. Six of the eight advanced high-Zn lines tested were developed by IRRI and two lines were from PhilRice (Table 1).

Table 1 List of genotypes tested at NCT

S.N	Genotype	Code	Source
1	IR10M210	G1	IRRI
2	IR10M300	G2	IRRI
3	IR83317-54-1-2-3	G3	IRRI
4	IR84749-RIL-47-1-1-1-1	G4	IRRI
5	IR84841-17-3-1-2	G5	IRRI
6	IR84842-87-3-1-2-2	G6	IRRI
7	MS13	G7	Micronutrient check
8	PR38732-B-B-1	G8	PhilRice
9	PR38963(F3)-B-7-1	G9	PhilRice
10	PSBRc82	G10	Yield Check

G Genotype, IRRI International Rice Research Institute, PhilRice Philippines Rice Research Institute, NCT National Cooperative Test

Experimental design and layout

The experimental design used for each test location was a randomized complete block design (RCBD) with three replications. Seedlings were transplanted at 21 days with a spacing of 20 × 20 cm, and the plant population was 15 rows of 21 hills in each plot in three replications. Standard agronomic practices and appropriate control measures for weeds, insect pests, and diseases were followed. Fertilizers were applied at recommended rates for every season at each location: 120-30-30 (N-P-K) during the DS and 90-30-30 during the WS.

Phenotyping

Agronomic traits such as days to maturity (MT), plant height (HT), number of tillers (TN), yield (YLD), reaction to insect pests and diseases, and grain quality characteristics were recorded using standard protocols and the guidelines set by NCT for rice. For the analysis of grain Fe and Zn, 20 g of paddy samples were dehulled using a Satake dehuller and milled for 1 min using a K-710 mini-lab rice polisher. These machines were fabricated with rollers coated with polyurethane to prevent any metal contamination. Impurities like hulls and broken grains were removed. About 10 g of clean milled samples were used in the analysis using an X-ray fluorescence spectrometer (XRF), Oxford X-Supreme 8000, at IRRI. For every sample two

readings were taken and averaged over three field replicates from each location.

Statistical analysis

Analysis of variance (ANOVA) was computed for all traits and locations during WS and DS. A separate analysis was carried out for all the locations and respective seasons combined. During 2014WS and 2015WS, each year and location were combined and treated as an environment. This was also done during 2015DS and 2016DS. For the single season analysis genotypes were considered fixed and locations were considered random. While, the effects of season and location and the genotypes were considered to be fixed in MET analysis. The performance of the ten genotypes tested for two DS and WS was analyzed using various stability models such as yield-stability index (YSi), AMMI, and GGE biplot using R (R Core Team 2012) and PBTools software (Version 1.4, <http://bbi.irri.org/products>).

YSi stability model was said to be useful in the selection for yield and stability, simultaneously. It uses modified Shukla's method in identifying stability and variance and integrating this with yield (Kang 1993). This model used R using the stability.par script included in the agricolae package.

```
stability.par(data, rep = n, MSError = n, alpha = n, main = "entryname", console = TRUE)
```

AMMI analysis uses ANOVA and PCA for estimating stability and G × E interactions (Gauch 1988; Yan et al. 2007). The AMMI model used for the stability analysis is as follows:

$$Y_{ij} = \mu + \delta_i + \beta_j + \sum_{k=1}^K \lambda_k \delta_{ik} \beta_{jk} + \varepsilon_{ij}$$

where Y_{ij} = mean of a trait of i th genotype in j th environment; μ = the grand mean; δ_i = genotypic effect; β_j = environmental effect; λ_k = eigenvalue of Interaction Principal Components Axes (IPCA) k ; δ_{ik} = eigenvector of genotype k for PC k ; β_{jk} = eigenvector for environment j for PC k ; and ε_{ij} = error associated with genotype i in environment j .

The GGE biplot uses a sites regression linear bilinear model (Crossa et al. 2002). It displays both genotypic main effects as well as G × E interactions (GGE). The GGE model used is given below:

Table 2 Mean performance of the genotypes

Genotype	MT (days)		HT (cm)		TN		YLD (kg ha ⁻¹)		Zn (ppm)		Fe (ppm)	
	WS	DS	WS	DS	WS	DS	WS	DS	WS	DS	WS	DS
G1	111.6	111.5	102.1	90.4	13.3	15.8	3845.2	4470.0	19.2	20.1	3.5	3.1
G2	113.6	118.5	105.9	100.4	12.8	15.1	3940.1	5081.3	19.6	19.1	3.4	2.8
G3	113.8	115.9	101.2	102.6	11.1	12.8	2211.7	3716.0	21.0	22.9	3.2	3.2
G4	113.2	116.2	104.9	97.3	12.7	16.1	3665.4	4856.1	18.9	19.7	3.4	3.0
G5	116.0	119.0	97.9	97.7	12.2	14.5	3604.5	4699.9	19.5	18.0	3.5	3.2
G6	117.1	122.3	110.6	105.7	10.3	13.4	2509.1	3527.2	21.3	21.8	3.2	3.1
G7	110.9	112.1	81.9	75.7	14.0	17.5	3404.1	4302.5	19.3	19.0	3.7	3.5
G8	121.4	123.6	105.8	101.3	10.8	13.8	3195.5	4329.2	17.8	16.5	2.7	2.7
G9	121.8	122.6	108.1	97.8	13.9	16.2	4318.4	5838.2	15.2	14.5	3.1	2.7
G10	116.6	116.8	107.0	93.8	14.7	16.2	4434.5	5296.7	15.4	15.8	3.2	2.8
Mean	115.7	117.9	102.9	95.4	12.6	15.2	3503.3	4825.7	19.4	18.4	3.9	2.6
Min	90	82	58	60	5	7	370	1582	8.8	10.7	1.3	0.9
Max	143	142	135	120.8	24	28	7940	11,182	39.7	29.6	7.0	7.3
Variance	164.9	95.7	217.7	151.2	9.4	17.6	2,132,097	4,050,355	26.0	13.1	2.5	2.1
SD	12.8	9.8	14.8	12.3	3.1	4.2	1460.2	2012.6	5.1	3.6	1.6	1.5

MT days to maturity, HT plant height, TN Tiller Number, YLD grain yield, Zn grain Zn, Fe Grain Fe, G genotype, Min minimum, Max maximum, SD standard deviation, ppm parts per million

$$Y_{ij} - \mu_j = \sum_{k=1}^t \lambda_k \delta_{ik} \beta_{jk} + e_{ij}$$

The values of δ and β are different from those of AMMI analysis but they represent the same variables.

Results

Performance of high-Zn genotypes

Large phenotypic variations were observed among the genotypes for all the traits across locations and seasons (Table 2). Genotypic means ranged from 110.9 to 121.8 days for MT, 81.9 to 110.6 cm for HT, 10.3 to 14.7 for TN per hill, 2211.7 to 4434.5 kg ha⁻¹ for YLD, 15.2 to 21.3 ppm for grain Zn, and 2.7 to 3.7 ppm for Fe in WS. Genotypes G1 and G7 were early maturing, whereas G8 and G9 were late maturing. Genotype G6 was the tallest and had the lowest number of productive tillers per hill, whereas G7 was the shortest. Genotype G10 had the highest number of productive tillers per hill and was the highest yielder, followed by yield check G9, G2, and G1. However, the first two high-yielding lines were the lowest in terms

of grain Zn. On the other hand, the genotypic means for the DS ranged from 111.5 to 123.6 days for MT, 75.7 to 105.7 cm for HT, 12.8 to 17.5 for TN per hill, 3527.2 to 5838.2 kg ha⁻¹ for YLD, 14.5 to 22.9 for grain Zn, and 2.7 to 3.5 ppm for Fe. Similar observations were made during DS with G1 and G7 as the early maturing, G6 being the tallest while G3 had the lowest number of productive hills. Genotypes G9 and G10 were high yielding lines but with the lowest grain Zn.

Analysis of variance

The combined ANOVA for all agronomic traits and grain Zn during the WS and DS is presented in Tables S2 and S3. The ANOVA showed significant genotypic effects and G × E interactions for all the traits. The AMMI analysis of variance for YLD and Zn in the WS and DS and in combined analysis also showed that genotype, environment, and G × E interactions were all significant (Table 3).

Table 3 AMMI analysis of variance for YLD and Zn

Source of variation	DS						WS					
	MS-YLD	TS	VE (%)	MS-Zn	TS	VE (%)	MS-YLD	TS	VE (%)	MS-Zn	TS	VE (%)
ENV	84,079,372	***	64.34	108.8	***	25.3	32,265,966	***	40.48	553.9	***	66.5
REP	1,055,158	***	1.85	2.6	*	1.5	562,530	ns	1.59	4.6	**	1.3
GEN	12,831,642	***	12.62	126.2	***	52.8	14,258,400	***	20.12	110.7	***	17.1
ENV:GEN	3,076,441	***	21.19	9.8	***	20.5	1,674,068	***	18.9	14.1	***	15.2
PC1	8,611,584	***	66.65	19.8	***	60.1	3,841,265	***	50.99	33.5	***	65.4
PC2	2,376,629	***	15.94	8.7	***	22.2	2,169,897	***	25.2	9.9	***	16.7
PC3	1,366,695	***	7.76	5.5	***	11.6	924,258.7	***	9.2	7.9	***	11.2
PC4	1,027,880	***	4.77	2.6	Ns	4.2	763,504	*	6.33	3.0	Ns	3.5
PC5	899,623.4	*	3.25	1.7	Ns	1.9	643,503	ns	4.27	1.7	Ns	4.0
PC6	433,602.2	ns	1.12	–	–	–	445,378.4	ns	2.22	1.3	Ns	0.8
PC7	331,976.7	ns	0.51	–	–	–	533,218.8	ns	1.77	1.7	Ns	0.7
PC8	–	–	–	–	–	–	7387.436	ns	0.01	–	–	–
PC9	–	–	–	–	–	–	–	–	–	–	–	–
Residuals	370,113	–	–	1.4	–	–	348,281	–	–	2.0	–	–
Combined												
MS-YLD		TS		VE (%)		Zn		TS		VE (%)		
66,808,354		***		64.62		348.9		***		56.0		
794,355		***		1.63		3.8		***		1.3		
26,097,928		***		14.20		224.1		***		24.9		
2,244,984		***		19.54		12.3		***		17.8		
6,463,967.7		***		47.99		29.4		***		46.9		
2,584,308.4		***		17.59		18		***		26.0		
1,825,128.5		***		11.29		9.2		***		11.9		
1,547,522.8		***		8.62		7.3		***		8.3		
1,042,373.2		***		5.16		3.0		ns		2.9		
861,504.4		***		3.73		2.1		ns		1.8		
719,549.9		*		2.67		1.9		ns		1.3		
566,514.3		ns		1.75		1.1		ns		0.6		
486,367.2		ns		1.20		1.0		ns		0.4		
358,555		–		–		1.7		–		–		

AMMI additive main effects and multiplicative interaction, MS mean sum of squares, TS test of significance, VE variation explained, DS dry season, WS wet Season, ENV environment, REP replication, GEN genotype, PC principal component

***Significant at $P < 0.0001$; **Significant at $P < 0.01$; *Significant at $P < 0.05$

Stability analysis

The performance of the genotypes and the effect of $G \times E$ interactions can be further explained by stability models like Kang’s stability index, AMMI, and GGE biplot models. These models will enable to identify stable high-Zn biofortified rice lines and thoroughly characterize environments for the

suitability and adaptation of high Zn lines in multi-location trials.

Stability analysis using Kang’s stability index (Si)

Results of stability analysis for all the traits using Kang’s stability model is presented in Table 4. The

positive Ysi ranking indicates the stable performance of genotypes for different traits across the locations. Three genotypes for MT, five genotypes each for YLD and Zn, six genotypes for Fe; and seven genotypes each for HT and TN were stable in WS. Similarly four genotypes for HT, five genotypes each for MT and YLD; six, seven and nine genotypes were stable for Zn, TN and Fe respectively in DS. In the combined analysis four genotypes each for MT, HT and Zn; five genotypes for YLD and seven genotypes each for TN and Fe were highly stable. Among the genotypes, G2 was superior for all six traits; G1, G6, and G9 were superior for four traits; and G3, G4, G5, G7, G8, and G10 were superior for three traits during DS. Similarly, G2, G9 and G10 was superior for five traits; G1 and G5 were superior for four traits; G4 was superior for three traits; G6, G7, and G8 were superior for two traits each; and G3 was superior for only one trait during WS.

The Ysi ranking showed the stability in performance of the genotypes across environments and seasons. Genotype G10 had the highest rank during WS whereas G2 was the most stable during DS. The overall ranking of genotypes for all the traits derived from the sum of stability indices showed that genotypes with the highest score during the WS were G10 followed by G6, G9, G2, G1, G5, and G4, whereas G2, G9, G1 = G5, G7, G10, and G4 = G8 were the top stable performers during the DS. Genotypes G2, G9 = G10, and G4 performed best in combined season analysis. This result showed that G2 performed consistently in terms of YLD and grain Zn and has been identified as a promising high-Zn breeding line.

Grouping of test environments

Environments grouped together denote positive correlations and may have same effect on the expression of the trait. Two distinct environment groupings (g1: E3, E4, E5 and g2: E1, E2, E6, E7, E8, E9) were observed for YLD in WS (Fig. 1a) and three groups (g1: E3; g2: E4; g3: E1, E2, E5, E6, E7) in DS (Fig. 1b). While, four groups in WS (g1-E1, E2, E3; g2-E4, E5; g3: E6, E7; g4-E8) and three groups in DS (g1: E1, E2, E3; g2:E4, E5; g3:E6) were observed for grain Zn (Fig. 2a, b).

The environment groupings for MT, HT, TN and Fe during WS are provided in the Figures S1–S4. Three environment groups each were observed for MT (g1-

E3; g2-E4, E6; g3-E1, E2, E5), HT (g1-E1, E2, E5, E6, E7; g2-E4, E8; g3-E3, E9), TN (g1-E1, E2, E6, E7, E8, E9; g2-E3, E4; g3-E5) and Fe (g1-E1, E6; g2-E2, E5, E8 and g3-E3, E4, E7). Similarly, the grouping of genotypes based on different traits during DS is presented in the Figures S5–S8. Three distinct groupings were observed for MT (g1-E1, E2; g2-E3, E5, E6; g3-E4), HT (g1-E1, E2; g2- E4, E6; g3-E3, E5, E7, E8) and TN (g1-E1, E2, E4, E6; g2-E5, E7, E8; g3-E3) and four groupings were observed for Fe (g1-E6; g2-E4; g3-E5; g4-E1, E2, E3).

Discriminating environments which are denoted by long vectors in the biplots provides information about performance of a particular genotype. The most discriminating environments during WS were Batac, Munoz, and Iguig for YLD and Fe, Batac and Iguig for HT and TN, while Iguig, Munoz, and Negros for Zn. During DS, Munoz and LB were the most discriminating sites for HT, TN, Zn and Fe; while Iguig and Munoz were found to be discriminating for MT and YLD. However, Negros was the least discriminating environment for YLD for both seasons.

The representativeness of a test environment can also be interpreted using GGE biplots. This is based on the relative distance to the Average-Environment Axis (AEA), the line that passes through the mean coordinates of all the test environments as represented by the small circle at the end of the arrow, which denotes the ideal test location. Munoz was the most representative test environment for MT, HT, Zn and YLD for both seasons while Iguig was the most representative environment for Fe, Zn and HT during WS.

Test environments that are both discriminating and representative were said to be ideal environments for selecting generally adapted genotypes. Environments that are discriminating but non-representative were ideal for specifically adapted ones. Munoz was the most ideal test environment for generally adapted genotypes while Batac and Iguig were for specifically adapted genotypes. For micronutrient traits, Munoz was ideal for generally adapted genotypes while Negros, Iguig and Batac for specific adapted genotypes for Zn and Fe.

Genotype response to wider adaptation

The AMMI analysis results are interpreted on the basis of AMMI biplot in which the graph is plotted with the main effect and first multiplicative axis term (PC1) for

Table 4 YSi ranking and significance of stability variance of genotypes per season

Genotype	MT			HT			TN			YLD			Zn			Fe			YSi sum			
	WS	DS	C	WS	DS	C	WS	DS	C	WS	DS	C	WS	DS	C	WS	DS	C	WS	DS	C	
G1	-9	-10	-9	3+	-9	-9	8+	5+	9+	9+	4+	2+	2+	-6	10+	2+	6+	7+	5+	11	7	0
G2	-7	5+	-6	4+	10+	11+	7+	6+	6+	2+	6+	3+	3+	3+	7+	9+	7+	2+	3+	16	36	26
G3	-6	-5	-4	-2	8+	0+	-7	-10	-9	-10	-9	-10	-10	-9	5+	5+	3+	8+	7+	-31	-3	-11
G4	-8	-7	-8	6+	-2	-1	6+	5+	5+	-1	0	1+	1+	2+	1	0+	-1	4	4+	4	1	1
G5	-1	6+	-3	-9	-3	-7	3+	3+	3+	6+	-1	-1	-1	1+	-7	-5	9+	9+	11+	9	7	-2
G6	3+	3+	3+	5+	5+	5+	-10	-9	-10	-9	-10	-9	-9	-10	4+	4+	-6	1	-2	27	-6	-9
G7	-10	-9	-10	-10	-10	-10	2	5+	5+	-5	0	-5	-5	-8	6+	-2	12+	12+	13+	-19	4	-9
G8	4+	5+	5+	1	3+	4+	-5	-7	-8	-6	1+	-7	-7	-7	0	-8	-10	-10	-10	-23	1	-24
G9	5+	4+	4+	8+	0	2+	7+	10+	6+	4+	5+	5+	5+	5+	-10	-6	-7	1	-8	22	10	3
G10	0	-4	1+	8+	-7	-3	9+	7+	8+	5+	4+	4+	4+	4+	-1	-9	4+	3	2	30	2	3
Trait mean	115.7	117.9	116.8	102.9	95.4	99.4	12.6	15.2	13.8	3503.3	4825.9	4125.7	19.4	18.4	19.0	3.9	2.6	2.6	3.3			
YSi mean	-2.9	-1.2	-2.7	1.4	-0.5	-0.8	2.0	1.5	1.5	-0.5	0	-1.7	1.0	1.5	-1.0	1.7	4.6	2.5				
LSD (0.05)	0.6	1.4	0.738	2.5	1.9	1.543	0.9	0.9	0.649	282.0	290.7	195.6	0.7	0.6	0.494	0.3	0.3	0.3	0.202			

YSi: yield stability index, WS wet season, DS dry season, C combined season analysis, MT days to maturity, HT plant height, TN Tiller number, YLD grain yield, Zn grain Zn, Fe grain Fe, LSD least significant difference

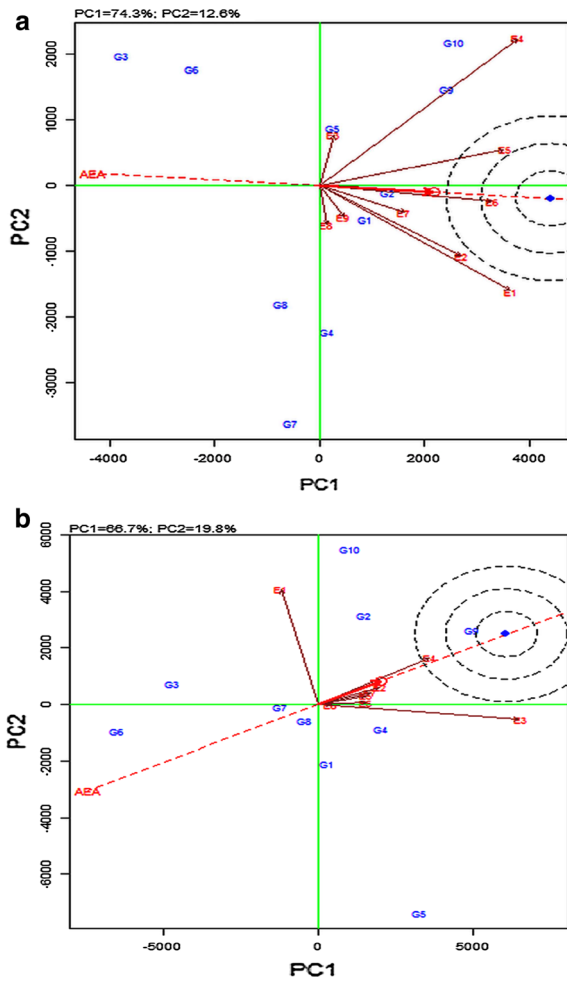


Fig. 1 a GGE-Environment biplot for YLD during WS. b GGE-Environment biplot for YLD during DS

both genotypes and environments. The greater value (either positive or negative) of the first principal component axis (PC1) indicates that genotypes are specifically adapted to a particular test environment or group of similar environments. Genotypes with PC1 scores close to zero are usually widely adapted and they are considered more stable in their performance across all the test environments.

In the WS, AMMI biplot showed 51% goodness of fit for YLD and 67.1% for Zn. Genotypes with high mean yield values include G10, G9, and G2, while G5 was close to the zero PC1 score line, making them the most stable with wider adaptation to the test environments (Fig. 3a). In terms of grain Zn, G3, G6, and G2 = G5 had high Zn values, with G4, G5 and G2 as the most stable genotypes (Fig. 3b).

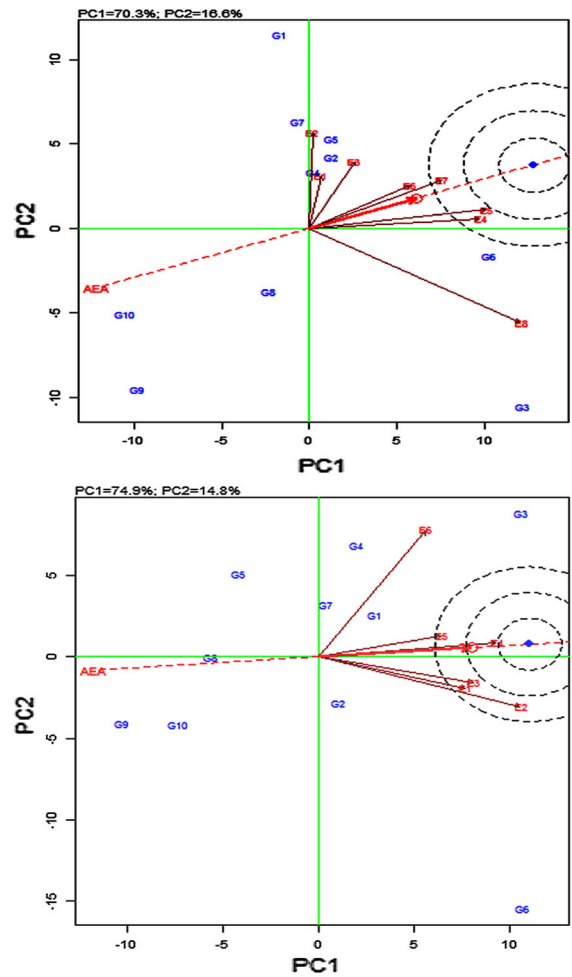


Fig. 2 a GGE-Environment biplot for Zn during WS. b GGE-Environment biplot for Zn during DS

The AMMI biplot showed goodness of fit of 66.6% for YLD and 58.9% for Zn during the DS. Among the genotypes, G9, G10, and G2 showed high yield with high main additive effects (Fig. 4a). G2 is relatively close to the zero PC1 score line with above mean grain yield values, thus have wider adaptation to the test environments. On the other hand, G3, G6 and G1 were genotypes with high grain Zn values (Fig. 4b). G1 and G2 were found to be the most stable and had wider adaptation, whereas G6 was found to be the most unstable.

The rankings and stability performance of the genotypes using GGE biplots for YLD and Zn are shown in Fig. 1a, b. In the WS, the first two principal components (PC1 and PC2) contributed 86.9% to the total variation for YLD with G10 and G9 as ideal

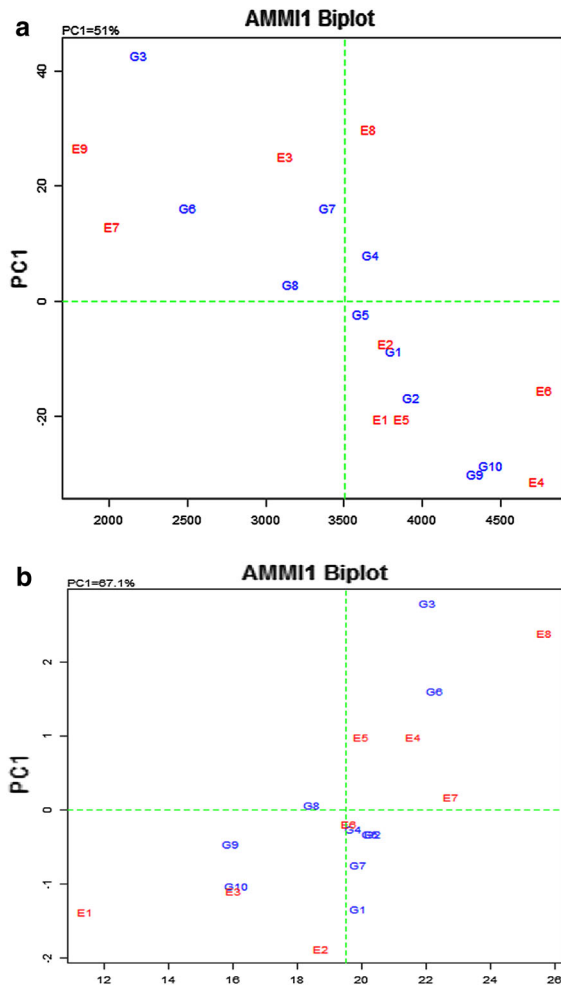


Fig. 3 **a** AMMI biplot for YLD during WS. **b** AMMI biplot for YLD during DS

genotypes having the highest mean yield, followed by G2, which was also considered the most stable genotype due to its close proximity to AEA. Genotype G3 was considered unstable and the poorest yielder. Similar result was observed in the DS (Fig. 1b); with G9 and G10 are the most ideal genotypes in terms of yield. Genotypes G2 and G4 were also high yielders and stable performers. G6 and G3 had below-average yield and were considered unstable genotypes together with G5.

In terms of grain Zn trait, G6 was the most ideal genotype, followed by G3 during the WS (Fig. 2a). Although these lines were high in grain Zn, they were relatively unstable due to their distant proximity to the AEA. G9 had the lowest grain Zn values, followed by

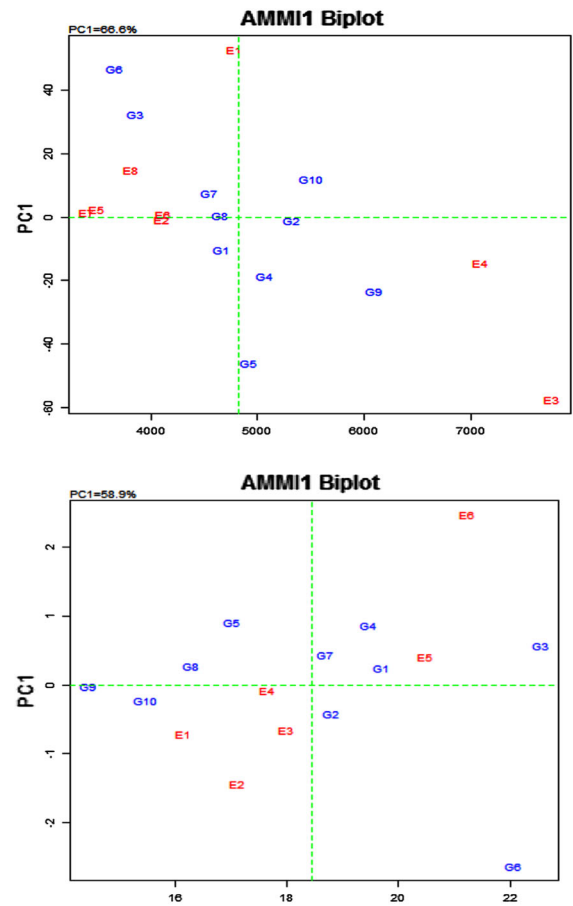


Fig. 4 **a** AMMI biplot for Zn during WS. **b** AMMI biplot for Zn during DS

G10 and G8. On the other hand, G2 and G4 were the most stable performers across environments and had grain Zn values above average. Differential expression of grain Zn levels was also observed during the DS (Fig. 2b).

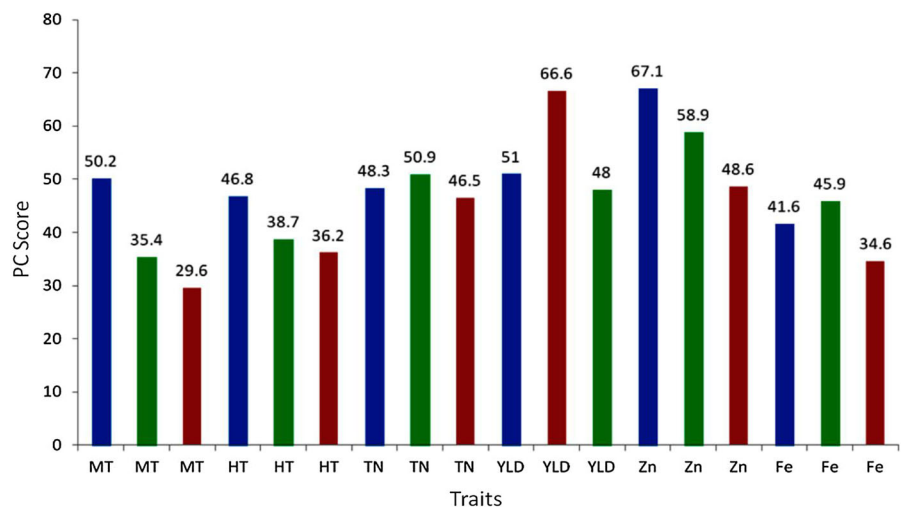
The AMMI biplots showed comparable result with that of the GGE in terms of stability ranking. In the combined season analysis similar results were also observed, with G9 and G10 as the highest yielder followed by G2 and G1 with G2 as the most stable (Fig S9). Whereas G3, G6, G1, G4, and G2 had high grain Zn (Fig S10). The principal components (PC1 and PC2) contributed 48 and 48.6% for the total variation for YLD and Zn, respectively (Fig. 5). Overall, the GGE and AMMI biplots showed that G9, G10, and G2 consistently performed well for both seasons in terms of YLD, while G3 and G6 were considered poor performers. G9 was the most ideal genotype for YLD

as it out yielded the check variety. Although G9 and G10 performed well in terms of YLD, these genotypes had the lowest Zn values. However, G2 was the most stable performer across different environments and seasons for both YLD and Zn traits. It also outperformed the two checks for both traits during the wet and dry season, making it the first released high-Zn variety in the Philippines.

Genotype response to specific adaptation

The graphical presentation of the “what-won-where” function of the GGE biplot makes use of polygons from which the genotypes situated on the vertices may either perform the best or worst in a particular environment. Genotypes located on the vertices may be considered as the winner or the poorest genotype in a given sector. Based on the results, the biplot for YLD during the WS showed that G10 was the winner genotype in E4, and G7 in E8 and E9. On the other hand, G9 was the winner for most environments (E5, E6, E7, E2, and E1), followed by G2 and G1 (Fig S11). During DS, G10 was the winner in E1, while G9 performed the best among the rest of the environments (Fig S12). For Zn, G1 was the winner genotype in E1, E2, and E3, G6 in environments E4, E5, E6, and E7, and G3 was selected as the best in E8 during the WS (Fig S13). Similar results were also observed during DS, in which G3 and G6 were again chosen as winner genotypes in E4, E5, and E6, and in E1, E2, and E3, respectively (Fig S14).

Fig. 5 PC1 scores for different traits in WS, DS and combined analysis. *MT* days to maturity, *HT* plant height, *TN* Tiller Number, *YLD* grain yield, *Zn* grain Zn, *Fe* Grain Fe



Correlation among the traits

In total, there were 45 correlations among six traits from the WS, DS, and combined analysis (Table 5). Out of these correlations, 23 were significant ($P < 0.05$), 8 of them were positively significant and 15 were negatively significant. Some of the interesting and positive correlations were between MT and HT, between TN and YLD in both the DS and WS, and between Fe and Zn only in the DS, while significant negative correlations were observed between Fe and MT, TN and HT, Fe and HT, and YLD and Zn during both the DS and WS; between Zn and MT and TN and Zn during the WS; and between TN and HT and YLD and Fe during the DS. Over all, the correlation for Zn between DS and WS was highly significant (0.924**).

Diversity analysis among the genotypes

The mean data for all six traits from both the DS and WS were used for estimating phenotypic diversity, and genotypes were clustered into different groups (Fig S15). At a significant threshold of $> 60\%$ diversity, all ten genotypes were grouped into three subgroups. Subgroup1 had two genotypes (G3 and G6), subgroup 2 had two genotypes (G9, and G10) and group 3 had a maximum of six genotypes (G1, G2, G4, G5, G7 and G8). The results clearly showed that all the yield checks were grouped into one group, and most of the IRRI-bred materials were grouped into one group. The two most promising genotypes, G1 (IR10M210) and G2 (IR10M300), were clustered together along with

Table 5 Correlation among different traits

Traits	Season	MT	HT	TN	YLD	Zn
HT	WS	0.568*				
	DS	0.711*				
	Combined	0.653*				
TN	WS	- 0.226	- 0.301			
	DS	- 0.483	- 0.807**			
	Combined	- 0.396	- 0.602*			
YLD	WS	0.121	0.087	0.839**		
	DS	0.117	- 0.145	0.617*		
	Combined	0.113	- 0.059	0.770**		
Zn	WS	- 0.539*	- 0.215	- 0.665*	- 0.797**	
	DS	- 0.372	0.167	- 0.479	- 0.817**	
	Combined	- 0.457	0.006	- 0.575*	- 0.839**	
Fe	WS	- 0.846**	- 0.694*	0.474	0.140	0.314
	DS	- 0.633*	- 0.544*	0.126	- 0.579*	0.595*
	Combined	- 0.812**	- 0.748*	0.300	- 0.252	0.503*

MT days to maturity, HT plant height, TN Tiller number, YLD grain yield, Zn grain Zn, Fe grain Fe

**Significant at $P < 0.01$; *Significant at $P < 0.05$

micronutrient check G7 (MS13). The overall results indicate that genotypes sharing a common parentage performed similarly in different environments.

Performance of the most stable high-Zn rice genotype, IR10M300

The NCT of rice established by the PhilRice began evaluating Zn-biofortified rice lines under the special purpose rice category during 2014. The first set of eight high-Zn rice lines (six from IRRI and two from PhilRice) was nominated and was evaluated along with the micronutrient check MS13 (IR68144-2B-2-2-3) and yield check PSBRc82 over four seasons during 2014WS (four sites), 2015DS (four sites), 2015WS (five sites), and 2016DS (four sites). Based on the overall performance of eight high-Zn rice lines for yield, agronomic traits, and grain Zn traits, G2 (IR10M300) performed better both in terms of yield ($4690.3 \text{ kg ha}^{-1}$) and grain Zn (18.3 ppm). In comparison with PSBRc82, IR10M300 had an average Zn increment of 4.3 ppm across all sites; however, it had lower yield of 450 kg ha^{-1} than the yield check. During 2014WS, the yield of IR10M300 was significantly higher than that of MS13 and comparable with that of PSBR82 across locations. In 2015DS, its yield was significantly higher than that of MS13 in Nueva

Ecija and Los Banos and comparable with that of PSBRc82 in Negros and Cagayan, whereas, in 2015WS, its yield was significantly higher than that of MS13 and PSBRc82 in Cagayan and Negros, respectively. In comparison with the previously released biofortified rice variety MS13 developed for its high Fe content, IR10M300 had 849 kg ha^{-1} more yield and consistently produced a 20% yield advantage during the DS and WS and across seasons, and had comparable grain Zn levels. Considering other agronomic traits, the performance of IR10M300 matches that of the commonly grown PSBRc82 in flowering date, plant height, and tiller count (Fig. 6). It has moderate blast, sheath blight, and green leafhopper resistance (Table S4). The grain quality traits of both IR10M300 and PSBRc82 are similar in intermediate amylose content, gel consistency, and gelatinization temperature (Table S5); sensory and cooking quality traits were also similar between these two genotypes (Table S6).

Discussion

Breeding for grain Zn is a complex process because of the polygenic inheritance of grain Zn and yield, and both are highly influenced by environmental factors

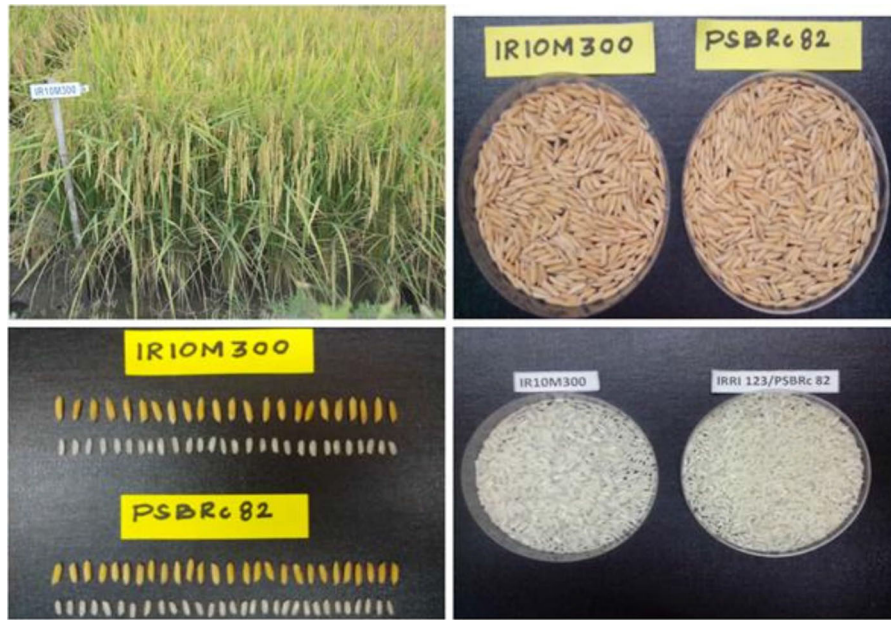


Fig. 6 Plant and grain type of IR10M300 and PSBRc82

(Gregorio et al. 2000; Wissuwa et al. 2008; Chandel et al. 2010; Swamy et al. 2011; Anuradha et al. 2012; Sarla et al. 2012; Rerkasem et al. 2015). Stable performance of rice genotypes for grain Zn and yield is essential for the successful development and release of high-Zn rice varieties. Multi-location evaluation and selection of stable genotypes for different traits are an integral part of the varietal release process in many countries (Bishaw and Van Gastel 2009). Although presence of $G \times E$ interactions is quite a challenge for breeders in evaluating lines in MET, the use of statistical models such as GGE, AMMI, and YSi statistic helps plant breeders to understand the performance of genotypes in different environmental conditions and allows selection of the most ideal genotypes for a particular environment or group of environments (Kang 1993; Gauch and Zobel. 1997). These models are also useful in identifying mega-environments and categorizing specific and generally adapted genotypes. Selection of the best performing and most stable genotypes will help in recommending them for commercial cultivation by farmers in a target environment (Anputhas et al. 2011).

The AMMI and GGE analyses showed significant genotype, environment, and $G \times E$ effects for Zn and YLD across seasons and locations. This is an

indication of the wide genetic background of the genetic materials as well as the diversity of test locations used. Earlier studies have also reported significant genotypic and $G \times E$ effects for yield, yield components, and grain micronutrients in rice (Chandel et al. 2010; Rerkasem et al. 2015; Suwanto and Nasrullah 2011; Ajmera et al. 2017).

There was a significant positive correlation between Fe and Zn and MT; and HT, while YLD and Zn were negatively correlated. Similar results have also been reported from earlier studies (Norton et al. 2010; Gao et al. 2006), whereas some reports show positive or non-significant correlations between YLD and Zn for both traits (Gangashetty et al. 2013; Sathisha 2013). So, it is possible to select for these two traits simultaneously or independently to develop high-Zn rice varieties with high yield potential. However, the selection of high-Zn donor lines with acceptable yield potential and designing an appropriate breeding strategy, selection schemes, and evaluation procedures are highly essential for the successful development and release of high-Zn rice varieties (Swamy et al. 2016; Suwanto and Nasrullah 2011).

Kang's stability ranking explains stability in the performance of genotypes across environments. In general, all the genotypes showed a better

performance for most of the traits during the DS vis-à-vis the WS. Within and between seasons, and in terms of trait combinations, the genotypes showed a differential response for different traits and relative ranks varied. Based on the overall ranking of genotypes for all the traits derived from the sum of stability indices, G2, G9 = G10, and G4 were the top stable performers. Overall, Kang's stability analysis results showed that G2 performed consistently in terms of YLD and Zn content and has been identified as a promising high-Zn breeding line. The yield stability index has been successfully used to identify rice genotypes with stable yield under different conditions (Nassir and Ariyo 2011; Balakrishnan et al. 2016; Oladosu et al. 2017).

The GGE biplot for different traits was able to show the relationship among the test environments and discriminating ability of a test environment based on genotype performance in different environments. Strong negative correlations between the environments for different traits reveal the presence of significant $G \times E$ interactions, while consistently non-discriminating sites will not provide any additional information on $G \times E$ interactions such sites can be excluded from trials to save cost and time. Our results showed that the Negros site was the least discriminating environment for YLD for both seasons. However, this site is good for screening pest and disease resistance, and soil nutritional deficiencies.

Munoz site is located in the most productive rice-growing areas of the Philippines. It was found to be the most representative and most discriminating environment; such locations usually depict mega-environment (Blanche and Myers 2006). Thus, Munoz can be considered as the most ideal environment for testing and identifying widely adopted high-Zn rice genotypes. Conversely, in Iguig, genotype performance was different from the average performance of genotypes from other locations, so this site was found to be suitable for specifically adapted genotypes for yield and Zn. The "what-won-where" GGE biplots clearly show the adoptability of genotypes for different traits. The genotypes falling on the vertices of the polygons in the GGE biplots indicate their level of performance in a particular environment (Yan and Tinker 2006).

The AMMI biplot results showed results comparable with those of Kang's stability rankings and GGE biplots. $G \times E$ interactions accounted for 51% and 66.6% of the total variation for YLD during the WS

and DS, respectively, while they accounted for 67.1% and 58.9% of the total variation for Zn during the WS and DS, respectively. Overall, the GGE and AMMI biplots showed that G9, G10, and G2 consistently performed well for both seasons in terms of YLD, while G3 and G6 were considered poor performers. G9 was the most ideal genotype for YLD as it out yielded the check variety. Although G9 and G10 performed well in YLD, these genotypes were lowest in Zn values. However, G2 was the most stable performer across different environments and seasons for both YLD and Zn values. It also outperformed the two checks for both traits during the wet and dry season.

The AMMI analysis of high-Fe genotypes over eight environments in Indonesia showed significant environment (74.4%), genotype (5.6%), and genotype \times environment interactions (19.7%) for Fe concentration in rice grains. The analysis identified genotype "Barumun" as the most stable for Fe concentration, and environment "Cilongok" was the best representative and most discriminating environment (Suwanto and Nasrullah 2011). Similarly, Ajmera et al. (2017) identified stable genotypes for Fe and Zn concentrations from multi-location evaluation of 37 diverse genotypes. Significant $G \times E$ interaction for mineral contents was observed among a set of rice germplasm. Soil pH and phosphorus were found to influence Fe content, while soil Zn status and electrical conductivity influenced Zn content in rice grains (Pandian et al. 2011). Wissuwa et al. (2008) reported that soil Zn status was the major factor that determined grain Zn in rice, followed by genotype and Zn fertilizer application.

Stability analysis using Kang's stability index, AMMI, and GGE consistently identified the stable genotypes. This indicates the accuracy of the different methods and their usefulness in understanding $G \times E$ interactions for complex traits and in identifying genotypes with wider or specific adaptability. We were able to identify G2 (IR10M300) as the most stable genotype across different environments and seasons, showing high yield and high grain Zn. It is comparable with PSBRc82 in terms of yield and it outperformed the micronutrient check, MS13, in terms of grain Zn and yield. This line has been released for commercial cultivation as "High-Zinc Rice 1 (NSICRc460)" for cultivation in the Philippines (Fig. 6). It represents the first of a series of Zn-

biofortified varieties developed through conventional breeding practices in the Philippines.

Considering the amount of genotype and environment interactions for both yield and Zn, and in comparison with the first generation of high-Zn rice released in other countries, NSICRc460 represents the first generation of high-Zn rice released for commercial cultivation in the Philippines. This will help to improve the Zn nutrition of Filipinos and create broader awareness among farmers, consumers, and the scientific community of the importance of high-Zn rice and the high-Zn rice project undertaken at IRRI and PhilRice for nutritional improvement. In addition, several high-Zn rice lines are currently in various stages of testing with improved grain Zn and yield.

The first series of high-Zn rice varieties released in other countries such as Bangladesh and India also had similar concentrations of Zn and yield value. BRRI dhan 62, the first Zn variety released in Bangladesh, had an average yield of 3.5 to 4.5 t ha⁻¹ with varied Zn concentration up to 19 ppm. Likewise, the high-Zn rice released in India, “Chhattisgarh Zn Rice-1,” had yield of 4.5 t ha⁻¹ with varied concentrations of grain Zn. A recent study showed that total absorbable Zn increased with increased dietary Zn (Zyba et al. 2016). The moderate increase in intake of Zn (4 mg day⁻¹) that is normally found in most Zn-biofortified crops could increase the total absorbable Zn, leading to increased concentrations of DNA repair proteins and improved DNA repair (Zyba et al. 2016). So, the High-Zinc Rice 1 released in the Philippines having an additional 4.5 ppm of Zn can have significant health benefits. Efforts are being made to widely disseminate the release of high-Zn rice in the Philippines and plans are being made to multiply and distribute high-Zn rice seeds to farmers in the Philippines.

Conclusions

Genotypes with high mean performance in terms of Zn and yield are the primary interest in Zn biofortification breeding. In this study, GGE, AMMI, and Ysi statistic were used to evaluate the stability of high-Zn rice lines among test locations used in varietal testing in the Philippines. IR10M300 showed a consistent performance for YLD, grain Zn content, and other agronomic traits. It has been successfully released for commercial cultivation in the Philippines as “High-

Zinc Rice 1”. In addition, the findings on the discrimination and representativeness of an environment are useful inputs for future high-Zn varietal evaluations.

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Authors' contributions MAIA analyzed the data and prepared the draft manuscript, AA and GILD assisted in draft preparation, ECA and TP were involved in conducting the experiments and gathering the data, and RR and BPM S selected the genotypes, designed the experiments, and edited the manuscript. All the authors have read and approved the manuscript.

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