Indian J. Genet. Plant Breed., (2023); 83(2): 157-167
https://doi.org/10.31742/ISGPB.83.2.1
ISSN: 0975-6906
www.isgpb.org

RESEARCH ARTICLE

Grain γ-oryzanol and its constituent compounds show high genetic variability, diversity and significant site x genotype interactions in rice (*Oryza sativa* L.)

Swarnadip Ghosh, Haritha Bollinedi*, S. Gopala Krishnan, Prolay K. Bhowmick, Nagarajan M.[1], K. K. Vinod, Ranjith K. Ellur and Ashok K. Singh

Abstract

Among the dietary antioxidants that counteract oxidative stress to avert the incidence of non-communicable diseases (NCDs), γ-oryzanol, a unique antioxidant in rice shows potential health benefits. Although rice germplasm exhibits significant variation for γ-oryzanol content, much less is studied on the influence of environment (E) and genotype-environment interaction (GEI) on the γ-oryzanol content in rice bran. Evaluated under three sites, in a combined analysis of variance, a set of 18 genotypes showed significant effect of environment, genotype, and GEI on the γ-oryzanol and its constituent compounds. A large proportion of total phenotypic variance was found due to genotype, indicating high heritability of the trait. GEI is the second major contributor to the phenotypic variance of γ-oryzanol and its components, while the contribution of environment was found to be the least. CSR 23 was the superior genotype for γ-oryzanol content and stability based on AMMI, GGE biplot and WAASB stability models analyzed. Besides CSR 23, genotypes such as DHMAS-70G-164-29, Chittimutyalu and HUR-200-57-1 were identified as stable and high γ-oryzanol producing lines. These superior, stable lines can be used as potential donors for improving γ-oryzanol content in rice. The present study assumes importance as the first report of GEI for total γ-oryzanol and its five components.

Keywords: AMMI, antioxidants, GGE biplot, γ-oryzanol, rice, stability

Introduction

Non-communicable diseases (NCDs), including cancer, diabetes, cardiovascular diseases, chronic respiratory diseases etc., are the leading cause of death globally, accounting for 41 million of the total 57 million fatalities (World Economic Forum 2022). Studies have shown that the modern lifestyle associated with unhealthy diets, reduced physical activity, and exposure to a wide range of harmful chemicals, environmental pollutants etc. is responsible for the build-up of reactive oxygen species (ROS) in the body. The ROS interacts with biomolecules such as proteins, lipids and DNA, causing their degeneration and thereby triggering oxidative stress (Gutteridge and Halliwell 1992; Young and Woodside 2001; Willcox et al. 2004). The oxidative stress further acts as a predisposing factor for the development of NCDs (Alberti et al. 2005). Antioxidants, mitigate oxidative stress and prevent the onset/progression of NCDs by neutralizing the free radicals through the donation of electrons (Abuajah et al. 2015).

As a major cereal crop globally, rice is a predominant dietary energy source for more than half of the world's population (Khush 2005). Besides carbohydrates, rice grain contains several components of benefit to human health.

Division of Genetics, ICAR-Indian Agricultural Research Institute, New Delhi 110 012, India

[1]ICAR-Indian Agricultural Research Institute, Rice Breeding and Genetics Research Centre, Aduthurai 612 101, Tamil Nadu, India

***Corresponding Author:** Haritha Bollinedi, Division of Genetics, ICAR-Indian Agricultural Research Institute, New Delhi 110 012, India, E-Mail: haritha.agrico@gmail.com

How to cite this article: Ghosh S., Bollinedi H., Krishnan G.S., Kumar S., Bhowmick P.K., Nagarajan M, Vinod K.K., Ellur R.K. and Singh A.K. 2023. Grain γ-oryzanol and its constituent compounds show high genetic variability, diversity and significant site X genotype interactions in rice. Indian J. Genet. Plant Breed., **83**(2): 157-167.

Source of support: ICAR, Govt. of India

Conflict of interest: None.

Received: Oct. 2022 **Revised:** March 2023 **Accepted:** April 2023

Among these, γ-oryzanol is an important antioxidant compound found in rice grains. It is a mixture of ferulic and caffeic acid esters of sterols and triterpene alcohol (Lerma-García et al. 2009). Although this group was reported to include 25 different components, about 95% of γ-oryzanol is composed of five steryl ferulate compounds viz., cycloartenyl ferulate (oryzanol A), 24-methylenecycloartanyl ferulate (oryzanol C), campesteryl ferulate, β-campesteryl ferulate and campestanyl ferulate (Akihisa et al. 2000; Xu et al. 2001; Miller and Engel 2006). Besides, other minor constituents such as sitosteryl ferulate, sitostanyl ferulate and stigmasteryl ferulate are present. Several previous works have reported antioxidative, anti-diabetic, anti-cancerous, anti-inflammatory and hypolipidemic properties of γ-oryzanol (Yasukawa et al. 1998; Akihisa et al. 2000; Juliano et al. 2005; Berger et al. 2005). Due to their extensive health benefits, γ-oryzanol has a great demand in the market, and is currently valued at about Rs. 1000 to 5000 per 100 capsules. In 2020, the global market size of γ-oryzanol was about 14.8 thousand tons and is expected to reach 20.6 thousand tons by the year 2027 (Global Industry Analysts 2021). As γ-oryzanol is accumulated predominantly in the bran layers of the rice grain, rice bran oil (RBO) is a rich source of γ-oryzanol. RBO contains 1-2% γ-oryzanol (Scavariello and Arellano 1998) besides its major constituens such as oleic, linoleic, palmitic, stearic and linolenic acids (Sahu et al. 2019). Owing to antioxidant properties imparted by γ-oryzanol, tocopherols, tocotrienols, and other phenolic compunds, RBO is acclaimed as a 'healthy oil'. Due to its antioxidative properties, γ-oryzanol prevents the rancidity of constituent lipids of RBO, improves the rice bran's stability, and extends the storage time of unpolished rice kernels (Bollinedi et al. 2021, 2022).

Understanding and estimating the differential genotypic response under different environments is one of the fundamental activities to establish the success of plant breeding interventions. Genotype by environment interaction (GEI) renders the genotypes to perform differently across multiple locations and years. The confounding effect of large GEI creates serious concerns in selection as well as in identifying and recommending genotypes for wider cultivation. Partitioning the trait variation into genotype (G), environment (E) and GEI components are therefore mandatory in plant breeding experimentation leading to the development of improved crop genotypes. Various biometric models like additive main effect and multiplicative interaction (AMMI) (Gauch 1988, 2006) and genotype and genotype-by-environment interaction (GGE) biplot analyses (Yan and Kang 2003; Yan and Tinker 2006) have been widely used for the analysis of GEI (Balakrishnan et al. 2016; Poli et al. 2018; Inabangan-Asilo et al. 2019). Recently Olivoto et al. (2019) have developed an integrated stability statistic combining AMMI and best linear unbiased predictor (BLUP) models. This statistic, known as the weighted average of absolute scores of BLUPs (WAASB) is derived from the singular value decomposition (SVD) of the predicted GEI effect matrix from a linear mixed-effect model.

Despite its importance, much less has been studied in rice to improve γ-oryzanol content. Particularly, the information on genotypic variability for various γ-oryzanol components and their expression levels under various environments is conspicuously lacking. To address this, the present study reports the GEI and genotypic stability and adaptability of a selected set of rice genotypes for γ-oryzanol and its components under three contrasting environments.

Materials and methods

Experimental locations

The field experiments were conducted during the *Kharif* (wet) season of 2021 at three different locations (sites) *viz*., New Delhi, Aduthurai and Rakhra. The sites were designated respectively as E1, E2 and E3. The experiment at New Delhi was conducted at the research farm of ICAR-Indian Agricultural Research Institute (IARI) (28°38'N; 77°10'E; 222m), while that at Aduthurai took place at IARI-Rice Breeding and Genetics Research Centre in Tamil Nadu (11°00N; 79°28'; 18m). The Rakhra experimental farm was located at the IARI-Collaborative Outstation Research Centre (IARI-CORC) in Patiala, Punjab, India (30°22'N; 76°15' E; 254m). The selected sites represented three different agroecological zones in India having diverse climatic and edaphic conditions.

Experimental material and design

A set of 18 Indian rice accessions (G1-G18) that included cultivated varieties, advanced breeding lines and landraces were field evaluated at all three sites by adopting the randomized complete block design (RCBD) with two replications. Nursery was prepared on a raised seedbed and 25 days old seedlings were transplanted at 20 × 15 cm spacing in the puddled main field. Each entry was grown in three rows of 2 m in length. Recommended package of practices and appropriate precautionary measures were taken to control the incidence of pests and diseases. At maturity, plants were harvested, seeds were threshed and dried below 13% moisture content and stored under aseptic conditions.

Phenotyping

For Extraction and quantification of γ-oryzanol the rice grains stored for 90 days were dehulled using Satake THU35B dehuller (Satake, Japan) and the brown rice was crushed using an Ez-lyzer tissue homogenizer (Genetix, India). For the extraction of γ-oryzanol from the brown rice samples, a solvent extraction method as described by Bollinedi et al. (2022) was followed. In brief, 50 mg of brown rice samples were transferred into 1-mL of high-performance

liquid chromatography (HPLC) grade methanol taken in a 2 mL Eppendorf tube, followed by vortex mixing for 1-minute. The samples were then incubated in a water bath at 60°C temperature for 1-hour. Subsequently, the incubated samples were centrifuged at 825 g for 10 minutes at room temperature. The supernatant was collected into 10 mL clean tubes and the extraction process was repeated twice using the residue. The supernatants thus collected were pooled and evaporated under a stream of N_2 and the samples were further dissolved in 1-mL HPLC grade methanol and filtered through a syringe filter having 0.22 µ pore size before injecting into HPLC.

A reversed phase high performance liquid chromatography (RP-HPLC) system (Waters Pvt. Ltd.) equipped with Waters 600 controller, a 717 plus autosampler and a 2998 photodiode array (PDA) detector was employed for the separation and quantification of individual components of γ-oryzanol. An XBridge™ C18 chromatography column (4.6 × 250 mm, inner diameter of 5 µm) was used for the separation of individual components of γ-oryzanol. The mobile phase was set in gradient pump mode using four different HPLC grade solvents: methanol, acetonitrile, 2-propanol and 1% aqueous acetic acid. The flow was set initially with methanol, acetonitrile, 2-propanol and 1% aqueous acetic acid at 45:45:5:5 (v/v) for 3 minutes and altered with methanol, acetonitrile and 2-propanol at 70:25:5 (v/v) for next 5 minutes. The flow was again reversed to the initial phase and ran for 6 minutes. Γ-oryzanol was detected using the PDA detector at 325 nm wavelength. The concentration of individual constituents was estimated from their relative abundance using the γ-oryzanol standard (Sigma Aldrich Co., USA).

Statistical analyses

The descriptive statistics, including mean, range, standard deviation, and coefficient of variation, were determined using Microsoft Excel® 2019. A combined analysis of variance (ANOVA) for γ-oryzanol content and its constituents for all three locations was carried out in STAR 2.0.1 (IRRI 2014 a, b). The homogeneity of variances was tested using Bartlett's test (Bartlett 1954). Stability analysis was carried out using the AMMI model, GGE biplot and WAASB (Olivoto et al. 2019) incorporated within the R package *metan* (Olivoto and Lúcio 2020).

The AMMI analysis followed the model, $Y_{ij}=\mu+g_i+e_j+\sum_{k=1}^{t}\gamma_n\beta_{in}\alpha_{ij}+\rho_{ij}+\varepsilon_{ij}$, where Y_{ij} is the mean trait value of the i^{th} genotype in the j^{th} environment, μ is the grand mean, g_i is the genotypic effect of the i^{th} genotype, e_j is the environmental effect of the j^{th} environment, n is the maximum number of interaction principal component axes (IPC) in the model to characterize the pattern and magnitude of the interaction between i^{th} genotype and j^{th} environment, γ_k is the eigenvalue of the k^{th} IPCA, β_{in} is the eigenvector for i^{th} genotype for PC *n*, α_{ij} is the eigenvector for the j^{th} environment for PC *n*, ρ_{ij} is the AMMI residue and ε_{ij} is the error with i^{th} genotype in the j^{th} environment. The genotypes showing the highest levels of stability for the content of total γ-oryzanol and its components were identified based on their closeness to the origin point of the AMMI 2 biplot.

The GGE biplot analysis used the model, $Y_{ij}^{*}=\sum_{k=1}^{t}\gamma_k\beta_{ik}\alpha_{ij}+\varepsilon_{ij}$ where Y_{ij}^{*} in the adjusted mean by subtracting the mean of the j^{th} environment from the performance of the i^{th} genotype in the j^{th} environment ($Y_{ij}^{*}=Y_{ij}-\mu_j$), γ_k is the eigenvalue of the k^{th} IPC, β_{ik} is the eigenvector for i^{th} genotype for PC *k*, α_{ij} is the eigenvector for the j^{th} environment for PC *k* constructed using genotype means from each environment and ε_{ij} is the error with i^{th} genotype in the j^{th} environment.

Finally, WAASB index was computed based on SVD of BLUPs considering all the IPCAs by the formula, $WAASB_i=\sum_{k=1}^{p}|IPC_{ik}\times EP_k|/\sum_{k=1}^{p}EP_k$ where $WAASB_i$ is the weighted average of absolute scores of the i^{th} genotype; $IPCA_{ik}$ is the score of the i^{th} genotype in the k^{th} interaction principal component axis (IPCA) and EP_k is the explained variance of the k^{th} IPC for $k = 1$ to p, considering p=min(g−1; e−1).

Results

Analysis of variance

Bartlett's test did not provide sufficient evidence of the heterogeneity of variance among the sites for the traits analyzed. A combined analysis revealed that site, genotype and GEI (site x genotype) components of variation were significant for all the traits studied (Table 1). The genotypic component was the largest among the various sources of variation. The proportion of total variance due to genotypes ranged from 50.7% (campesteryl ferulate) to 65.9% (24-methylenecycloartanyl ferulate). The second largest component was GEI explaining a variance fraction ranging from 13.9% for 24-methylenecycloartanyl ferulate to 21.9% for cycloartenyl ferulate. The contribution of the environment was found to be the least. The coefficient of variation (CV) for total γ-oryzanol content was 7.9%. Among the constituent compounds, cycloartenyl ferulate showed the lowest CV of 6.5% while sitostanyl ferulate showed the highest CV of 28.1%. Based on averages, the total γ-oryzanol content of 198.4 mg kg^{-1} was constituted by the five major components studied. 24-methylenecycloartanyl ferulate was the most prominent component (44%) of γ-oryzanol, followed by campesteryl ferulate (~22%) and cycloartenyl ferulate (~18%). The fourth major constituent was β-sitosteryl ferulate (~14%). The remaining component, sitostanyl ferulate was present minimally, accounting for ~2% of the total γ-oryzanol.

Site-wise variation in γ-oryzanol and its components

Descriptive statistics of γ-oryzanol and its five components across the three sites are given in Table 2. Widest variation for total γ-oryzanol and its constituents was observed in

Rakhra, except for cycloartenyl ferulate. The highest mean total γ-oryzanol content of 211.1 mg kg^{-1} was recorded at Aduthurai, which was closely followed by the recording at the Rakhra site. Among the genotypes tested, the site at Delhi recorded the lowest γ-oryzanol content of 79.0 mg kg^{-1}, while the highest content (347.3 mg kg^{-1}) was observed at Rakhra site. 24-methylenecycloartanyl ferulate remained the major constituent in all three locations, the maximum content of which was recorded at Aduthurai site (96.4 mg kg^{-1}). The highest cycloartenyl ferulate content was recorded in the Delhi site, which was closely similar to the content recorded from the other two sites. Other components, campesteryl ferulate and β-sitosteryl ferulate followed the general pattern among the sites, with Aduthurai recording highest followed by Rakhra and Delhi. (29.61 mg kg^{-1}). Cycloartenyl ferulate and sitostanyl ferulate contents showed no significant difference among all three test sites.

Genotypic performance for γ-oryzanol

Phenotypic mean values showed that among the three test sites, CSR 23 was distinctly different from other genotypes for having the highest total γ-oryzanol content and its constituent compounds (Table 3). CSR 23 contained a total of 316.3 mg kg^{-1} γ-oryzanol, and 128.6 mg kg^{-1} of 24-methylenecycloartanyl ferulate, the most prevalent fraction. For the remaining fractional compounds, this genotype was found to have 58.6 mg kg^{-1} of cycloartenyl ferulate, 66.7 mg kg^{-1}of campesteryl ferulate, 51.7 mg kg^{-1} of β-sitosteryl ferulate, and 10.7 mg kg^{-1} of sitostanyl ferulate. For 24-methylenecycloartanyl ferulate content, Chittimutyalu (129.88 mg kg^{-1}) and DHMAS 70G-164-29 (127.8 mg kg^{-1}) were at par with CSR23. WGL 23985 (51.1 mg kg^{-1}) was the only genotype found at par with CSR 23 for cycloartenyl ferulate. The next highest γ-oryzanol content was recorded in Chittimutyalu, DHMAS 70G-164-29 and HUR 200-57-1. Narendra Usar Dhan III recorded the panel's lowest total γ-oryzanol content (88.6 mg kg^{-1}).

Genotypic diversity for γ-oryzanol

Based on principal components, eighteen genotypes tested in the panel formed three distinct groups based on the content of γ-oryzanol and constituent components (Fig. 1). One of the clusters contained only CSR 23, the genotype with high amount of γ-oryzanol. The second cluster with five genotypes, Phunchi, Narendra Usar Dhan III, PRR120, PRR 103 and PRR 108 contained the genotypes that had the lowest amount of γ-oryzanol. The third cluster with 12 genotypes was intermediate to the first two clusters. Of the principal components, the first component accounted for 83.7% of the total variation in γ-oryzanol content with all the components contributing almost equally and significantly. The second and third components were defined majorly by cycloartenyl ferulate and campesteryl ferulate. The third component also had a contribution from 24-methylenecycloartanyl ferulate.

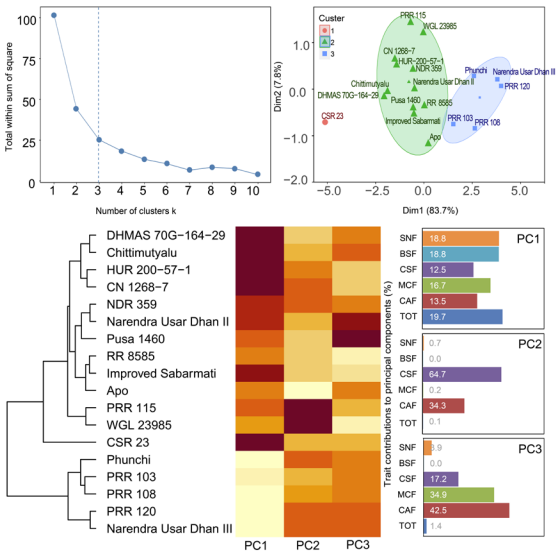

Fig. 1. Grouping of genotypes based on γ-oryzanol and its constituent compounds based on k-means clustering of principal component scores

Biplot analyses for genotypic stability and GE interactions

Additive main effects and multiplicative interaction

The ANOVA for the AMMI model revealed a high significance of the variance of the first two interaction principal

Table 1. Combined analysis of variance for γ-oryzanol and its components across three sites

Parameters	TOT	CAF	MCF	CSF	BSF	SNF
Var (Site)	6945.6**	30.7**	3164.4**	320.3**	143.1**	7.3**
Var (Genotype)	19760.1**	891.7**	5458.3**	481.1**	587.6**	46.3**
Var (Genotype x Site)	2917.3**	177.2**	576.4**	99.8**	113.0**	7.8**
Var (Residual)	245.1	5.2	50.0	9.5	6.1	1.6
CV (%)	7.9	6.5	8.1	7.2	8.8	28.1
Mean (mg kg^{-1})	198.4	35.4	87.2	43.0	28.3	4.5
Per cent constitution (%)	-	17.9	44.0	21.7	14.2	2.3

**significant at 1% level. Var = Variance; TOT = Total γ-oryzanol; CAF = Cycloartenyl ferulate; MCF = 24-Methylenecycloartanyl ferulate; CSF = Campesteryl ferulate; BSF= β-sitosteryl ferulate and SNF = Sitostanyl ferulate.

Table 2. Descriptive statistics of γ-oryzanol and its five components in three different sites

Traits	Statistics	Components (mg kg^{-1})		
		Delhi	Aduthurai	Rakhra
Total γ-oryzanol (TOT)	Mean ± SE	183.6 ± 14.3	211.1 ± 15.2	200.7 ± 16.6
	Range	79.0-318.0	95.5-287.4	91.3-347.3
Cycloartenyl ferulate (CAF)	Mean ± SE	36.3 ± 3.6	35.5 ± 3.0	34.5 ± 3.6
	Range	16.8-67.8	13.9-60.7	16.0-64.8
24-methylenecycloartanyl ferulate (MCF)	Mean ± SE	77.7 ± 7.2	96.4 ± 8.6	87.6 ± 7.6
	Range	24.0-126.2	30.1-142.9	26.7-143.0
Campesteryl ferulate (CSF)	Mean ± SE	39.6 ± 2.2	45.0 ± 2.2	44.5 ± 3.1
	Range	25.7-62.7	28.2-61.5	25.5-79.1
β-sitosteryl ferulate (BSF)	Mean ± SE	26.0 ± 2.5	29.6 ± 2.8	29.2 ± 3.0
	Range	11.9-50.1	11.6-45.6	11.1-59.3
Sitostanyl ferulate (SNF)	Mean ± SE	4.0 ± 0.7	4.5 ± 0.7	4.9 ± 0.9
	Range	0.2-11.1	0.3-8.5	0.4-12.6

SE = Standard error

components (IPCs), indicating significant GEI variation for all the traits (Table 4). IPC1 and IPC2 were found to account for 100% of the GEI for all the traits studied. The AMMI1 biplot (Fig. 2A) revealed that IPC1 for the total γ-oryzanol content, explained 69.9% of the total interaction variance, with IPC2 explaining the remaining proportion of 31.1%. The genotype CSR 23 having the highest mean γ-oryzanol content showed relatively better stability than at least eight genotypes from the panel. This genotype has shown high content of all the component compounds with relatively less influence by the site effects, except for campesteryl ferulate. Among the high γ-oryzanol genotypes, DHMAS 70G-164-29 followed by HUR 200-57-1 were identified as the most stable genotypes across the three environments since their IPCA1 scores remained very low. Among the γ-oryzanol low genotypes, PRR108, Narendra Usar Dhan III and PRR 120 showed high stability of performance across sites. Genotypes such as PRR 103 and CN 1268-7 with the highest IPCA1 scores were identified as the least stable genotypes. Similarly, for the constituent compounds, the proportion of total variance explained by IPCA1 ranged between 60.2% (campesteryl ferulate) and 79.6% (24-methylenecycloartanyl ferulate). The most stable genotypes for cycloartenol ferulate content were Chittimutyalu and Narendra Usar Dhan II of which the former had a higher content of the compound.

The most stable among high cycloartenyl ferulate genotypes was WGL 23985. For 24-methylenecycloartanyl ferulate content, DHMAS 70G-164-29 and Pusa 1460 recorded higher content combined with stability, while WGL 23985, Apo and RR8585 remained stable but with the average constitution of the ester. Apo, the genotype with the second highest content of campesteryl ferulate among the panel, showed better stability, followed by HUR 200-57-1. Two other genotypes, Narendra Usar Dhan III and PRR 120 showed high stability but with low content. CSR 23 showed the highest β-sitosteryl ferulate content and stability among the study panel. Although PRR 120, Narendra Usar Dhan III, and PRR 108 showed high stability for this constituent ester, their contents were the lowest among the panel. High content and stability for the sitostanyl ferulate content were observed in DHMAS 70G-164-29 followed by NDR 359. Comparing the environmental adaptation, genotypes with IPCA1 scores similar to the environments are considered to have superior performance in that particular environment. Better sites for total γ-oryzanol content were Aduthurai and Rakhra, to which genotypes such as CN 1268-7 and Chittimutyalu showed specific adaptation. The AMMI 2 biplots depicting the pattern of GEI among the test genotypes, showed a congregation of several genotypes towards the origin, indicating that these genotypes are highly stable as their IPCA1 scores and IPCA2 scores are closer to zero (Fig. 3).

GGE biplots

GGE biplots that combine the effect of genotype with GE component indicated that 96.3% of the total variation in γ-oryzanol was accounted for by the first two principal components (Fig. 2B). For the components, however, the variance explained ranged from 88.4% (campesteryl ferulate) to 96.5% (24-methylenecycloartanyl ferulate). The ranking based on genotype performance obtained from GGE biplots did not show substantial variation from the inference drawn from AMMI biplots. In the biplot for total γ-oryzanol content, the narrow angle between Aduthurai and Rakhra sites indicated a greater correlation of genotype performance in these sites. The 'which-won-where' view of the GGE biplot

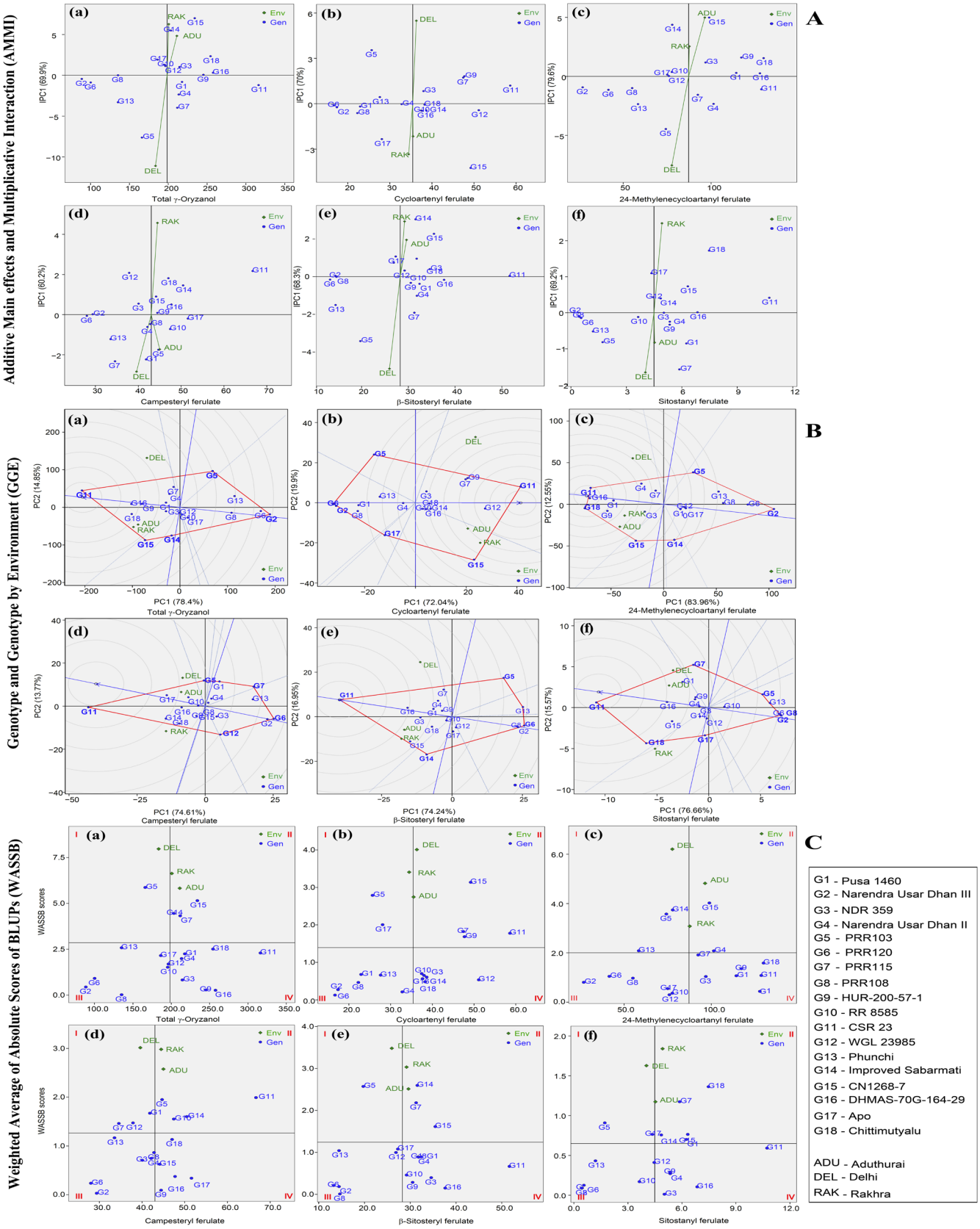

Fig. 2. (A) AMMI1, (B) GGE and (C) WAASB biplots showing GEI for γ-oryzanol and its components for 18 genotypes under three sites for γ-oryzanol and its components

Table 3. Mean performance of the genotypes across the three test environments

Genotype	γ-oryzanol and components (mg-1 kg)					
	TOT	CAF	MCF	CSF	BSF	SNF
Pusa 1460	217.7^{de}	22.9^{g}	114.3^{b}	41.8^{fg}	32.4^{de}	6.3^{b-d}
Narendra Usar Dhan III	88.6^{l}	17.4^{h}	26.9^{h}	29.4^{j}	14.4^{i}	0.4^{h}
NDR 359	214.5^{e}	38.0^{d}	96.8^{cd}	40.1^{gh}	34.6^{cd}	5.0^{d-f}
Narendra Usar Dhan II	213.5^{e}	32.8^{e}	101.4^{c}	42.1^{fg}	31.8^{ef}	5.4^{d-f}
PRR 103	166.1^{i}	25.7^{f}	74.2^{e}	44.7^{d-f}	19.7^{h}	1.7^{h}
PRR 120	100.1^{k}	16.7^{h}	41.6^{g}	28.0^{j}	13.3^{i}	0.6^{h}
PRR 115	211.6^{ef}	47.4^{c}	92.5^{d}	34.6^{i}	31.3^{ef}	5.9^{c-e}
PRR 108	135.3^{j}	22.3^{g}	55.1^{f}	42.8^{fg}	14.6^{i}	0.5^{h}
HUR 200-57-1	245.3^{bc}	47.7^{c}	117.2^{b}	44.5^{d-f}	30.5^{ef}	5.3^{d-f}
RR 8585	195.4^{f-h}	37.5^{d}	77.7^{e}	47.4^{c-e}	29.2^{fg}	3.6^{g}
CSR 23	316.3^{a}	58.6^{a}	128.6^{a}	66.7^{a}	51.7^{a}	10.7^{a}
WGL 23985	196.4^{f-h}	51.1^{b}	76.0^{e}	37.9^{h}	26.9^{g}	4.5^{fg}
Phunchi	135.3^{j}	27.6^{f}	58.4^{f}	33.6^{i}	14.4^{i}	1.2^{h}
Improved Sabarmati	203.4^{e-g}	38.7^{d}	77.9^{e}	50.5^{bc}	31.6^{ef}	4.9^{e-g}
CN 1268-7	234.1^{cd}	49.2^{bc}	98.9^{cd}	44.2^{f}	35.5^{bc}	6.3^{b-d}
DHMAS 70G-164-29	257.7^{b}	37.7^{d}	127.8^{a}	47.7^{cd}	37.6^{b}	6.9^{bc}
Apo	186.4^{h}	28.1^{f}	75.1^{e}	51.6^{b}	27.3^{g}	4.4^{fg}
Chittimutyalu	254.5^{b}	38.3^{d}	129.9^{a}	47.0^{de}	31.8^{ef}	7.5^{b}
LSD (p=0.05)	16.5	2.4	7.5	3. 3	2.6	1.3

This means superscripted with the same letters are statistically at par by the least significant difference (LSD) test. TOT = Total γ-oryzanol; CAF = Cycloartenyl ferulate; MCF = 24-Methylenecycloartanyl ferulate; CSF = Campesteryl ferulate; BSF = β-sitosteryl ferulate; SNF = Sitostanyl ferulate.

across all the test locations for total γ-oryzanol and all its components. This was followed by CN 1286-7, which showed relatively better performance under both Aduthurai and Rakhra, for total γ-oryzanol, cycloartenyl ferulate and 24-methylenecycloartanyl ferulate. Besides, CSR 23, Chittimutyalu also showed better performance across all the sites.

WAASB biplots

The WAASB biplots depicted the stable and high performing genotypes, isolating them, particularly in the fourth quarter (Fig. 2C). Accordingly, stable and high performing genotypes for total γ-oryzanol were CSR 23, DHMAS 70G-164-29, Chittimutyalu, HUR 200-57-1, Pusa 1460, NDR 359 and Narendra Usar Dhan II. Likewise, WGL 23985 was identified as the best for cycloartenyl ferulate content. The highest stability and yield combination was noticed for DHMAS 70G-164-29, followed by CSR 23 and Chittimutyalu for the second component, 24-methylenecycloartanyl ferulate. For campesteryl ferulate, Apo was found better in terms of stable content of the ester molecule. In the case of β-sitosteryl ferulate and sitostanyl ferulate, CSR 23 performed far superior to all other genotypes in the panel.

Discussion

Because of its excellent health benefits, improving RBO content in rice is recently recognized as a breeding objective because of its excellent health benefits. RBO is commercially marketed as an edible oil in Asia Pacific with China, India and Japan as the major producers (Pal and Pratap 2019). RBO contains ~2% γ-oryzanol, a major antioxidant. The nutritional benefits of RBO earned its popular name 'heart oil' in Japan. These RBO compounds present various health benefits, from acting as antioxidants to anti-neoplastic agents (Yasukawa et al. 1998; Oka et al. 2009; Kim et al. 2015). Oryzanols are commonly produced in the bran layers of the rice grain, in different quantities and with different constitutions

Table 4. Variance components of multiplicative interaction for γ-oryzanol and its components

Source	TOT	CAF	MCF	CSF	BSF	SNF
IPC1	3853.2** (69.9)	234.2** (70.0)	866.3** (79.6)	113.5** (60.2)	145.8** (68.3)	10.2** (69.2)
IPC2	1864.5** (31.1)	113.0** (30.0)	250.4** (20.4)	84.4** (39.8)	76.1** (31.7)	5.1** (31.8)

Values in parentheses indicate the proportion of variance explained by respective components. IPC = Interaction principal component; TOT = Total γ-oryzanol; CAF = Cycloartenyl ferulate; MCF = 24-Methylenecycloartanyl ferulate; CSF = Campesteryl ferulate; BSF = β-s itosteryl ferulate and SNF = Sitostanyl ferulate; ** $p < 0.001$.

showing the convex hull connecting genotype sitting on the vertices was used to identify superior genotypes as well as mega-environments. Although the biplots depicted the presence of two mega-environments, the grouping could lead to ambiguity considering the geographical locations of the sites under study.

In general, the genotype CSR 23 was found to be the vertex genotype demonstrating superior performance depending on the varieties (Huang and Ng 2011). Although recognized to be important in terms of human health benefits, detailed studies on variability, genetic diversity and GEI of γ-oryzanol content in rice genotypes remain scanty. This study, therefore, assumes importance as the first report integrating variability, diversity and GEI of γ-oryzanol production in rice.

Conduction of multi-environment trials (METs) is a

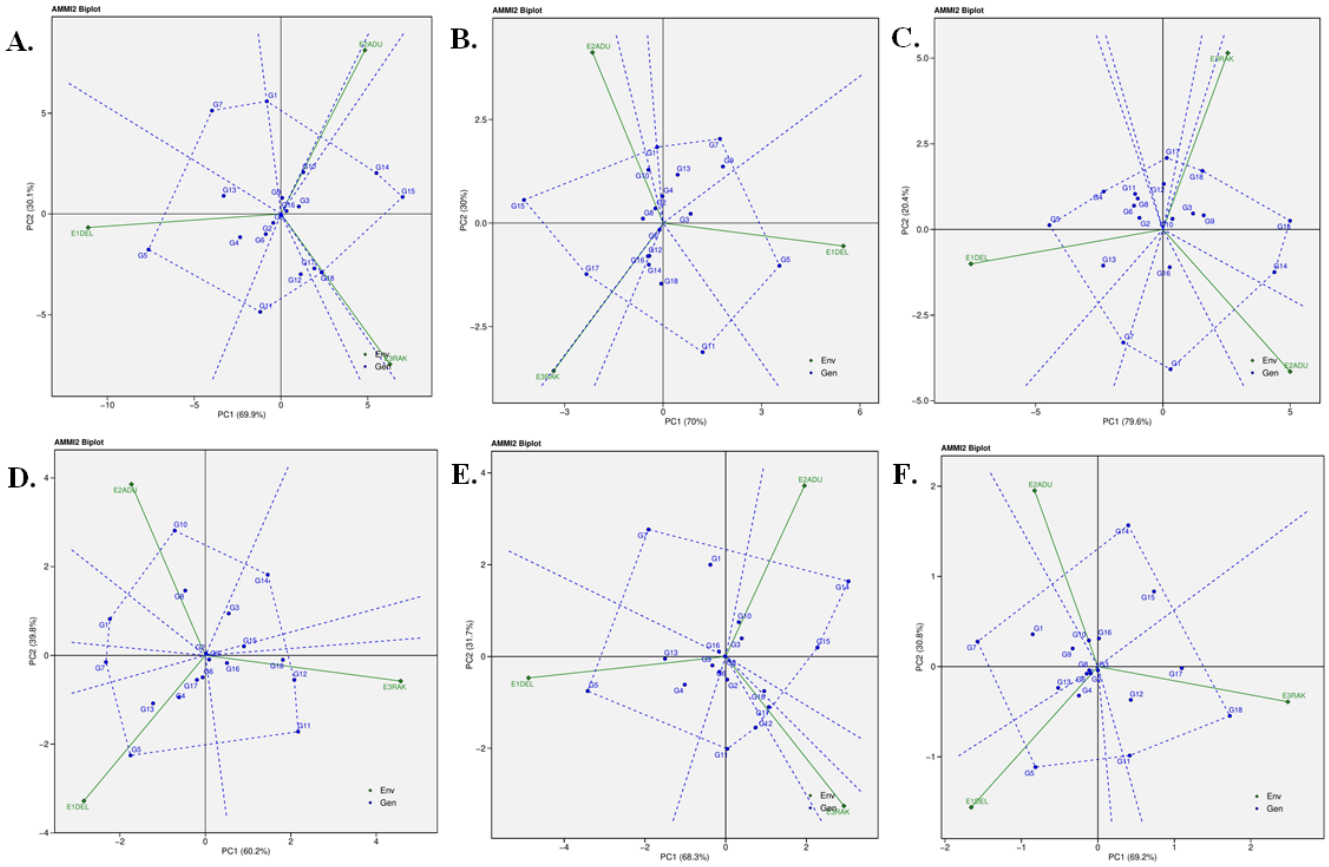

Fig. 3. AMMI 2 biplots of stability analysis for γ-oryzanol and its components, (A) total γ-oryzanol, (B) cycloartenyl ferulate, (C) 24-methylenecycloartanyl ferulate, (D) campesteryl ferulate, (E) β-sitosteryl ferulate, (F) sitostanyl ferulate

routine practice by plant breeders to understand the GEI, recommend genotypes to specific environments, and delineate the mega-environments (Olivoto et al. 2019). GEI enables us to understand the stability or consistency of trait expression under changing environments. Stability allows the breeder to develop widely adapted or specifically adapted varieties considering the magnitude of GEI. Further, understanding GEI is important in the development of climate-resilient rice varieties. There are several methods to estimate GEI from METs. Although, linear statistical models are computationally simple and easy to understand they suffer from robustness in GEI estimations. Conversely, several complex models that integrate linear models and multivariate approaches are currently available that can bring out concrete information about GEI, including genotype and environment behaviour and adaptation affinities. Most popular among these are biplot approaches, such as AMMI and GGE, and recently WAASB is being used as a novel unbiased approach (Olivoto et al. 2019; Vineeth et al. 2022). Although these methods are interrelated, they also offer specific advantages in investigations of GEI.

The partitioning of total phenotypic variation for γ-oryzanol content and its constituent compounds indicated that γ-oryzanol production can vary between sites, and shows considerable genotype x environment interactions. Therefore, the genetics of γ-oryzanol in rice can be concluded as quantitative and is under the regulation of several contributing genes. Although significant, the site variance was found to be a relatively nominal contributor to the total variance, indicating the scope for developing widely adapted genotypes acceptable to more diverse environments. Further, a large proportion of total phenotypic variance was found due to genotype, indicating relatively high heritability of the trait. Notwithstanding, the significance of GEI could not be ignored because it was the second highest contributor to the total variance after the genotypic effects. Since the genotypes responded differentially in different test environments (Shukla et al. 1972), it became essential to assess their performance stability. The pattern of diversity among the genotypes indicated that most of the common cultivars belong to the intermediate groups, as it was the largest cluster in the test panel. We have used principal components (PC) for diversity partitioning, as they are unbiased and uncorrelated orthogonal vectors enabling us to obtain the best dispersion of genotypes between the PC axes. The diversity pattern in

the study panel suggests that high γ-oryzanol genotypes are relatively lower in proportions, while low genotypes can be found relatively more frequently. Further, the constitution of γ-oryzanol indicated high content of oryzanol C (24-methylenecycloartanyl ferulate) in rice followed by campesteryl ferulate and oryzanol A (cycloartenyl ferulate).

In the AMMI approach, the GE component was stratified into the multiplicative interaction factor called IPCs. IPCs can easily picture the GEI pattern since the IPC1 accounts for the maximum variation in GEI, and IPC2 is the next highest. In this study, IPC1 accounted for more than 60% of the GE variation; hence, plotting the genotypes with observed means against IPC1 scores could provide an opportunity for simultaneous selection for the genotype performance with stability. The resultant AMMI1 biplot could reveal that CSR 23 is the winning genotype carrying high γ-oryzanol among the panel, with good stability in γ-oryzanol production across environments. CSR 23 is a salt tolerant semi-dwarf rice variety having medium slender grains released in India in 2004. It was developed by the International Rice Research Institute with the designation, IR52713-2B-8-2B derived from a three-way cross, IR64//IR4630-22-2-5-1-3/IR9764-45-2-2 (Singh and Gregario 2006). This line can be further used for breeding for RBO improvement in rice.

Notwithstanding, there are genotypes such as Chittimutyalu, DHMAS 70G-164-29 and HUR 200-57-1 which contained ~250 mg kg^{-1} γ-oryzanol) showing promise for further utilization. Chittimutyalu is a popular short grain aromatic rice from Southern India already known for high grain Zn and Fe content (Surekha et al. 2019). DHMAS 70G-164-29 and HUR 200-57-1 are breeding lines.

Since an effective partitioning of GE from genotype performance is unfeasible, the GGE biplot approach assumes both components together while assessing the individual performance. Since the environment is independently dealt with, the GGE biplot provides an evaluation of the environment itself. In the biplots, an average environment axis (AEA) runs through the origin and a hypothetical average environment in the direction of mean performance of the individual genotypes represented by an arrow. Test environments that are closer to the AEA are the most representative of the ideal environment, while the length of the environment vectors indicates their discriminating power. The genotypes are ranked along the AEA backward starting in the direction of the arrowhead. The selection of the winning genotypes is carried out from the 'which-won-where' view of GGE biplot. As identified from AMMI analysis, the genotype CSR 23 was identified as the top winning by GGE as it remained at the vertex position in the direction of AEA. For total γ-oryzanol, genotypes that ranked after CSR 23 were DHMAS-70G-164-29, Chittimutyalu and HUR-200-57-1. Better stability of these genotypes could be re-established by GGE method.

The sites that are representative of the ideal environment are considered favourable environments (Yan and Tinker 2006). Accordingly, in this study the three test sites could be ranked in the order of their representativeness as Aduthurai > Rakhra > Delhi for total γ-oryzanol, cycloartenyl ferulate and sitostanyl ferulate; Aduthurai > Delhi > Rakhra for campesteryl ferulate and β-sitosteryl ferulate; Rakhra > Aduthurai > Delhi for 24-methylenecycloartanyl ferulate. Interpreting the relationships among the sites (Yan and Rajcan 2002), the angles between Aduthurai and Rakhra locations were low for total γ-oryzanol, cycloartenyl ferulate, 24-methylenecycloartanyl ferulate and β-sitosteryl ferulate, indicating significantly similar performance between these two sites. Aduthurai and Delhi sites had a comparable expression for campesteryl ferulate and sitostanyl ferulate. The Rakhra site was most discriminative for all the traits, except for 24-methylenecycloartanyl ferulate content. Delhi location shared a larger angle with other sites, indicating the large diversity of this site from the other two.

WAASB index is a relatively new measure of genotypic stability proposed by Olivoto et al. (2019) that combines the dual advantages of the graphical view of AMMI biplots and the prediction accuracy of BLUPs. Like other methods, WAASB biplots also allow selecting genotypes based on the joint interpretation of productivity combined with stability. The advantage of WAASB is the use of GE BLUPs that provide better statistical prudence. Lower the WAASB score, higher would be the stability of the genotypes, and this score is plotted against mean trait values. Genotypes falling in the quarter where WAASB scores low and trait mean higher are identified as the superior genotypes with stable performance. In this method also, WAASB scores picked genotypes such as CSR 23, DHMAS-70G-164-29 and Chittimutyalu as superior genotypes for total γ-oryzanol content as identified by other models. A stable genotype may not necessarily be a high performing genotype.

γ-oryzanol is an important component of RBO that imparts antioxidant quality to the oil. This study could establish genetic variability and GEI for γ-oryzanol content and its components in a subset of random rice genotypes suggesting the scope of identifying superior rice accessions with higher levels of oryzanols. We could demonstrate that γ-oryzanol content in rice shows significant GEI when grown under varying environments. The study also suggests that component oryzanols vary by genotype. The genotypes with higher oryzanol components can be used for mapping genes responsible for each of the esters and those genotypes with higher stability and yield can be used for improving RBO quality in rice grains through breeding interventions.

Authors' contribution

Conceptualization of research and designing of the

experiments (HB); Contribution of experimental materials (HB, AKS, GKS); Execution of field/lab experiments and data collection (SG, PK, MG, RKE); Analysis of data and interpretation (HB, SG, KKV); Preparation of the manuscript (SG, HB, KKV).

Acknowledgment

The authors are grateful to Indian Council of Agricultural Research (ICAR) through the Consortium Research Project on Biofortification in rice. The first author duly acknowledged the Indian Council of Agricultural Research, New Delhi for providing post-graduate fellowship during the Master's degree programme.

References

Abuajah C. I., Ogbonna A. C. and Osuji C. M. 2015. Functional components and medicinal properties of food: a review. J. Food Sci. Technol., **52**(5): 2522-2529. https://doi.org/10.1007/s13197-014-1396-5.

Akihisa T., Yasukawa K., Yamaura M., Ukiya M., Kimura Y., Shimizu N. and Arai K. 2000. Triterpene alcohol and sterol ferulates from rice bran and their anti-inflammatory effects. J. Agric. Food Chem., **48**(6): 2313-2319. https://doi.org/10.1021/jf000135o.

Alberti K. G. M., Zimmet P. and Shaw J. 2005. The metabolic syndrome—a new worldwide definition. The Lancet, **366**(9491): 1059-1062. https://doi.org/10.1016/S0140-6736(05)67402-8.

Balakrishnan D., Subrahmanyam D., Badri J., Raju A.K., Rao Y.V., Beerelli K., Mesapogu S., Surapaneni M., Ponnuswamy R. and Padmavathi G. 2016. Genotype× environment interactions of yield traits in backcross introgression lines derived from *Oryza sativa* cv. Swarna/*Oryza nivara*. Front. Plant Sci., **7**: 1530. https://doi.org/10.3389/fpls.2016.01530.

Bartlett M. S. 1954. A note on the multiplying factors for various χ 2 approximations. Journal of the Royal Statistical Society. Series B (Methodological), 296-298. https://www.jstor.org/stable/2984057.

Berger A., Rein D., Schäfer A., Monnard I., Gremaud G., Lambelet P. and Bertoli, C. 2005. Similar cholesterol-lowering properties of rice bran oil, with varied γ–oryzanol, in mildly hypercholesterolemic men. Eur. J. Nutr., **44**(3): 163-173. https://doi.org/10.1007/s00394-004-0508-9.

Bollinedi H., Singh A. K., Singh N., Bhowmick P. K., K. K, V., and Ellur R. K. 2021. Genetic and genomic approaches to address rapid rancidity of rice bran. Crit. Rev. Food Sci. Nutr., **61**(1): 75-84. https://doi.org/10.1080/10408398.2020.1718598.

Bollinedi H., Singh N., Krishnan S.G., Vinod K.K., Bhowmick P.K., Nagarajan M., Ellur R.K. and Singh A.K. 2022. A novel LOX3-null allele (*lox3-b*) originated in the aromatic Basmati rice cultivars imparts storage stability to rice bran. Food Chem., **369**: 130887. https://doi.org/10.1016/j.foodchem.2021.130887.

Gauch Jr H. G. 1988. Model selection and validation for yield trials with interaction. Biometrics, 705-715. https://doi.org/10.2307/2531585.

Gauch Jr. H.G. 2006. Statistical analysis of yield trials by AMMI and GGE. Crop Sci., **46**: 1488-1500. https://doi.org/10.2135/cropsci2005.07-0193.

Global Industry Analysts Inc. 2021, April. Gamma Oryzanol, Global Market Trajectory & Analytics. (Article ID-5302729), Research and Markets. Available at: https://www.researchandmarkets.com/reports/5302729/gamma-oryzanol-global-market-trajectory-and.

Gutteridge J. M. and Halliwell B. 1992. Comments on review of free radicals in biology and medicine, by Barry Halliwell and John MC Gutteridge. Free Radic. Biol. Med., **12**(1): 93-95. https://doi.org/10.1016/0891-5849(92)90062-l.

Huang, S.H. and Ng, L.T. 2011. Quantification of tocopherols, tocotrienols, and γ-oryzanol contents and their distribution in some commercial rice varieties in Taiwan. J. Agric. Food Chem., **59**(20): 11150-11159. https://doi.org/10.1021/jf202884p.

Inabangan-Asilo M.A., Swamy B.M., Amparado A.F., Descalsota-Empleo G.I.L., Arocena E.C. and Reinke R. 2019. Stability and G× E analysis of zinc-biofortified rice genotypes evaluated in diverse environments. Euphytica, **215**: 61. https://doi.org/10.1007/s10681-019-2384-7.

Juliano C., Cossu M., Alamanni M. C. and Piu L. 2005. Antioxidant activity of gamma-oryzanol: mechanism of action and its effect on oxidative stability of pharmaceutical oils. Int. J. Pharm., **299**(1-2): 146-154. https://doi.org/10.1016/j.ijpharm.2005.05.018.

Khush G. S. 2005. What it will take to feed 5.0 billion rice consumers in 2030. Plant Mol. Biol., **59**(1): 1-6. https://doi.org/10.1007/s11103-005-2159-5.

Kim, H.W., Lim, E.J., Jang, H.H., Cui, X., Lee, S.H., Kim, H.R., Choe, J.S., Yang, Y.M., Kim, J.B. and Park, J.H. 2015. 24-Methylenecycloartanyl ferulate, a major compound of γ-oryzanol, promotes parvin-beta expression through an interaction with peroxisome proliferator-activated receptor-gamma 2 in human breast cancer cells. Biochem. Biophys. Res. Commun., **468**(4): 574-579. https://doi.org/10.1016/j.bbrc.2015.10.147.

Lerma-García M.J., Herrero-Martínez J.M., Simó-Alfonso E.F., Mendonça C.R., and Ramis-Ramos G. 2009. Composition, industrial processing and applications of rice bran γ-oryzanol. Food Chem., **115**: 389-404. https://doi.org/10.1016/j.foodchem.2009.01.063.

Miller A. and Engel K. H. 2006. Content of γ-oryzanol and composition of steryl ferulates in brown rice (*Oryza sativa* L.) of European origin. J. Agric. Food Chem., **54**(21): 8127-8133. https://doi.org/10.1021/jf061688n.

Oka, T., Fujimoto, M., Nagasaka, R., Ushio, H., Hori, M. and Ozaki, H. 2010. Cycloartenyl ferulate, a component of rice bran oil-derived γ-oryzanol, attenuates mast cell degranulation. Phytomedicine, **17**(2): 152-156. https://doi.org/10.1016/j.phymed.2009.05.013.

Olivoto T. and Lúcio A. D. C. 2020. metan: An R package for multi-environment trial analysis. Methods Ecol. Evol., **11**(6): 783-789. https://doi.org/10.1111/2041-210X.13384.

Olivoto T., Lúcio A. D., da Silva J. A., Marchioro V. S., de Souza V. Q. and Jost E. 2019. Mean performance and stability in multi-environment trials I: combining features of AMMI and BLUP techniques. Agron. J., **111**(6): 2949-2960. https://doi.org/10.2134/agronj2019.03.0220

Pal Y. P., and Pratap A. P. 2017. Rice bran oil: A versatile source for edible and industrial applications. J. Oleo Sci., **66**: 551-556. doi : 10.5650/jos.ess17061

Poli Y., Balakrishnan D., Desiraju S., Panigrahy M., Voleti S.R., Mangrauthia S.K. and Neelamraju S. 2018. Genotype×

Environment interactions of Nagina 22 rice mutants for yield traits under low phosphorus, water limited and normal irrigated conditions. Sci. Rep., **8**: 1-13. https://doi.org/10.1038/s41598-018-33812-1.

Sahu P.K., Mondal S., Sharma D., Sao R., Kumar V. and Das, B.K. 2019. Genetic insights into fatty acid components of traditional Indian rice (*Oryza sativa* L.) landraces from Chhattisgarh Indian J. Genet. Plant Breed., **79**: 651-657. https://doi.org/10.31742/IJGPB.79.4.2

Scavariello E.M. and Arellano D.B. 1998. Gamma-oryzanol: un importante componente del aceite de salvado de arroz. Arch Latinoam Nutr., **48**:7-12.

Shukla G. K. 1972. Some statistical aspects of partitioning genotype-environmental components of variability. Heredity, **29**(2): 237-245. https://doi.org/10.1038/hdy.1972.87.

Singh R. K. and Gegario G. B. 2006. CSR23: a new salt-tolerant rice variety for India. Int. Rice Res. Note. **31**: 16-18.

Surekha K., Babu M. P., Bhowmick M. K., Pandey A., Baskar A., Sireesha A., Rao D. S., Babu V. R. and Neeraja C. N. 2019. GenotypexEnvironment interaction on rice grain yield, iron and zinc concentration. IIRR Newslett. **17**: 3-4.

Vineeth T.V., Prasad I., Chinchmalatpure A.R., Lokeshkumar B.M., Kumar S., Ravikiran K.T. and Sharma P.C. 2022. Weighted average absolute scores of BLUPs (WAASB) based selection of stable Asiatic cotton genotypes for the salt affected Vertisols of India. Indian J. Genet. Plant Breed., **82**: 104-108. https://doi.org/10.31742/IJGPB.82.1.15

Willcox J. K., Ash S. L. and Catignani G. L. 2004. Antioxidants and prevention of chronic disease. Crit. Rev. Food Sci. Nutr., **44**(4): 275-295. https://doi.org/10.1080/10408690490468489.

World Economic Forum. 2022. Why the 21st century's biggest health challenge is our shared responsibility. *World Economic Forum Annual Meeting.* Available at: https://www.weforum.org/agenda/2019/01/why-the-21st-centurys-biggest-health-challenge-is-our-shared-responsibility/.

Xu Z., Hua N. and Godber J. S. 2001. Antioxidant activity of tocopherols, tocotrienols, and γ-oryzanol components from rice bran against cholesterol oxidation accelerated by 2, 2'-azobis (2-methylpropionamidine) dihydrochloride. J. Agric. Food Chem., **49**(4): 2077-2081. https://doi.org/10.1021/jf0012852.

Yan W. and Kang M. S. 2002. GGE biplot analysis: A graphical tool for breeders, geneticists, and agronomists. CRC press. https://doi.org/10.1201/9781420040371.

Yan W. and Rajcan I. 2002. Biplot analysis of test sites and trait relations of soybean in Ontario. Crop Sci., **42**(1): 11-20. https://doi.org/10.2135/cropsci2002.1100.

Yan W. and Tinker, N.A. 2006. Biplot analysis of multi-environment trial data: Principles and applications. Can. J. Plant Sci., **86**(3): 623-645. https://doi.org/10.4141/P05-169.

Yan W., Cornelius P. L. Crossa J. and Hunt L. A. 2001. Two types of GGE biplots for analyzing multi-environment trial data. Crop Sci., **41**(3): 656-663. https://doi.org/10.2135/cropsci2001.413656x.

Yasukawa, K., Akihisa, T., Kimura, Y., TAMURA, T., & TAKIDO, M. (1998). Inhibitory effect of cycloartenol ferulate, a component of rice bran, on tumor promotion in two-stage carcinogenesis in mouse skin. Biol. Pharm. Bull., **21**(10): 1072-1076. https://doi.org/10.1248/bpb.21.1072.

Young I. S. and Woodside J. V. 2001. Antioxidants in health and disease. J. Clin. Pathol., **54**(3): 176-186. http://dx.doi.org/10.1136/jcp.54.3.176.