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Genetic Structure and Markers -Trait Association Analyses for Fe-Toxicity Tolerance, Grain-Fe Content and Yield Component Traits in Rice

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Abstract

Genetic diversity, structure analysis and marker-trait association for Fe toxicity tolerance in rice were studied using 80 shortlisted germplasm lines and 30 selected microsatellite markers. A moderate level of genetic diversity was detected in the studied population. Principal component and Ward's clustering analysis distributed the genotypes into various spots and clusters on the basis of LBI scores and other traits. Among the shortlisted genotypes, two genotypes Kanchan, and Mahalaxmi produced grain yield of >4 t/ha consistently under the stress. STRUCTURE software grouped all the genotypes into 3 genetic structure groups. These structure groups corresponded well with the Fe toxicity tolerance in rice. The marker-trait association analysis showed association of Fe toxicity tolerance, grain-Fe content and yield component traits using both Generalized Linear Model (GLM) and Mixed Linear Model (MLM/ K+Q and model) analyzed by TASSEL 5 software. LBI showed significant associations with RM5897 and RM 206 by both the models. A novel QTL controlling Fe-toxicity was detected and named as *qFeTox2.1*.Marker RM 206 associated with QTL for Fe-tolerance on chromosome 12 is also validated. GML and MLM detected association of grain-Fe content with markers RM105, RM1278 and RM5897. The region in chromosome 2 from 2.88Mb to 10.3Mb has multiple QTLs and hot spot for Fe content in rice grain. QTL *qFe9.1*, controlling the grain Fe-content and co-localization of RM1278 with Fe toxicity tolerance are validated in the study. Grain number is strongly associated with marker RM5897 detected by both the models.

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Background

Iron (Fe) is considered as an essential micronutrient and is a constituent of many enzymes of rice plant. Shortage of this micronutrient affects rice plant growth, development and yield. The micronutrient acts as co-factor for enzymes in photosynthesis, nucleic acids synthesis, mitochondrial respiration, metal homeostasis, and structural constituent in proteins and chlorophyll [1-5]. Deficiency of this element in soil is a stress to the rice plant. Due to limitation of the micronutrient in the plant, this affects quality of foods also. Around 30% of the global populations are reported to be affected by iron-deficiency related health issues (http://www.who.int/nutrition/topics/ida/en/). The element is not available to rice plant at neutral and above soil pH. More available Fe in soil increases uptake and creates toxicity to the rice plants [6-16]. Iron accumulates in cell compartments and plant tissues and creates metabolism problems [17-19]. Iron deficiency and toxicity are two abiotic stresses to rice plant reported across the world. Fe toxicity is considered as a stress to the rice plants in Sierra leone, Burundi, Burkina Faso, Liberia, Nigeria, Togo, Srilanka, Senegal, Phillippines, Indonesia, Malaysia, Thailand, Vietnam, Malaysia, India, Senegel, Colombia, Benin, Ivory Coast, Niger, Gambia, Guinea, Guinea-Bissau, Liberia, Nigeria, Indonesia, Malaysia, Thailand, Vietnam [20-23]. Genetic variation for Fe-toxicity tolerance exists in rice germplasm. Gene(s)/QTLs responsible for this stress tolerance and development of robust markers for improvement of tolerance in high yielding rice varieties are urgently needed for increasing rice yield from the affected areas.

The genomic region controlling tolerance to Fe toxicity in rice is observed to be governed by many genes and highly complex in nature. Few quantitative trait loci (QTL) have already been reported from different mapping populations in rice [24-28]. As per earlier reports, chromosomal regions mainly located around 25 and 30 Mb on chromosome1 and on chromosome 3 between 0 and 5 Mb are responsible [22,26,27,29], but no strong marker contributing higher phenotypic variance for major locus been reported, validated and used in rice improvement programs. However, few reports are available based on bi-parental mapping populations. Association mapping using large number of genotypes may help for identifying more number of loci responsible for Fe toxicity tolerance in rice. Reports of transporter genes involved in toxicity tolerance has been well reported [30-36].

In the current investigation, we phenotyped 80 shortlisted rice genotypes under Fe-toxicity field condition for tolerance to iron toxicity tolerance. These genotypes were also genotyped with 30 polymorphic molecular markers to estimate the genetic structure and to detect possible association of markers with Fe-toxicity tolerance, grain-Fe content and yield component traits in rice to be useful in rice improvement for Fe-toxicity tolerance breeding program.

Materials and methods

Plant Material, experimental site and design

A total of 80 shortlisted rice (*Oryza sativa* L.) genotypes consisting of landraces and released cultivars maintained at ICAR-National Rice Research Institute, Cuttack were used for the investigation (Table 1). The genotypes were shortlisted based on late maturity duration suitable for lowland ecology. The experiment was conducted in an iron toxicity hotspot field at RRTTS, OUAT, Bhubaneswar during wet seasons, 2017 and 2018. The genotypes were transplanted in the sick plot adopting the field layout in randomized block design with three replications. The recommended package of practices for shallow lowland was followed to obtain a good crop. The initial Fe level was 202.5 ppm in the sick plot soil. The field was maintained under saturated anaerobic condition.

Phenotyping for Fe-toxicity tolerance under sick plot condition

Phenotyping of 80 rice lines was done by considering the parameters like days of 50% flowering, plant height, panicle length, number of grains per panicle, 1000-grain weight, grain yield, leaf bronzing index (LBI) and numbers of tillers/hill. These observations were recorded following the Standard Evaluation System of Rice [37]. LBI was recorded from three replications of each genotype. The genotypes with score 6 to 9 were considered susceptible, 4-5 moderately resistant, 1-3 resistant and 0 as immune to Fe toxicity. Analysis of variance for morphologic traits was performed as per the previous method followed in the earlier publication [38-39].

DNA isolation and molecular characterization

The genotyping work was taken up at Molecular Breeding Lab.1, ICAR-National Rice Research Institute, Cuttack, Odisha. Total genomic DNA was extracted from five week old plants of the rice germplasm lines and varieties following stepwise CTAB protocol [40]. PCR amplification was performed in a Gradient Thermal Cycler (Veriti, Applied BioSciences) following the standard procedure followed earlier [4-42]. The list of markers selected for the study was based on our previous GWAS results on grain Fe-content (Table 2). Thirty rice microsatellite markers distributed over all 12 chromosomes. The amplification products were loaded in 3% gel containing 0.8 µg/ml Ethidium bromide for electrophoresis in 1X TBE (pH 8.0). One lane was loaded with 50bp DNA ladder. The gel was run at 2.5V/cm for 4 hrs and photographed using a Gel Documentation System (SynGene). Data scored were analyzed on the basis of the presence or absence of amplified products for each genotype-primer combination. The molecular diversity parameters *viz*., number of alleles, gene diversity, allele frequency, polymorphic information index (PIC) and heterozygosis were computed using the program PowerMarker Ver3.25 [43]. The marker-trait association analysis was carried out by using TASSEL5 software [44].

Results

Phenotyping of germplasm lines under Fe-toxicity sick plot

The screening for Fe toxicity tolerance in 80 shortlisted genotypes was performed under Fe-toxic sick field showing Fe level of 202.5 ppm. The leaf bronzing symptoms were observed after 4 weeks and scored at 6 weeks after transplanting. The leaf bronzing index (LBI) scores in the genotypes varied from 1.0 to 7.0. Sixty genotypes showed LBI score of 1 to 3.0 in the field screening and grouped as tolerant to the abiotic stress. A score of 3.0 to 5.0 was observed in 14 genotypes categorizing them under the moderate group. The rest 6 tested genotypes had low tolerance to the toxicity (>5 SES score) and were classified as susceptible types to the stress. The genotypes producing higher grain yield and showing tolerance to the stress were considered as promising genotypes (Table 1). Among the genotypes, only two genotypes, Kanchan and Mahalaxmi were found to produce >4t/ha yield and tolerance to the stress consistently over years. The frequency of genotypes showing poor, moderate and high tolerance to the stress is depicted in Figure 1.

The biplot analysis using 9 agro-morphologic traits in the shortlisted genotypes revealed the presence of majority of the variations in the 2 principal components of the PCA. The first principal component accounted for 80.2 % variance showing eigen value of 1528. The component 2 exhibited 14.77% variance with an eigen effect of 281.5. The genotypes with high value of leaf bronzing were in the same quadrant revealing similar phenotypes to the toxicity response. The encircled area in the in the quadrant accommodates the desirable genotypes with low bronzing scores and better yield (Figure 2). The quadrant 3 possessed all the susceptible and moderate group genotypes with low tolerance to soil Fe-toxicity tolerance (Figure 2).

Genetic diversity and clustering

The genotyping was performed using 30 SSR polymorphic markers for estimating the diversity parameters in the studied germplasm lines for iron toxicity tolerance (Table 2). The details of genetic diversity parameters obtained with these 30 polymorphic markers are shown in Table 2. Wide variations in alleles ranging from 90bp to 310bp were observed. The major allele frequency ranged from 0.2759 (RM206) to 1.000 (RM556) exhibiting a mean value of 0.6443. The average polymorphic information content (PIC) of 0.3966 indicated a moderate level of diversity in the population. The maximum polymorphic information content of 0.6972 was observed in RM206 and minimum value of 0.000 was observed in RM556. The gene diversity average value of all the tested markers was observed to be 0.4564. RM206 showed maximum gene diversity while RM556 had minimum value amongst all markers in the 80 shortlisted rice genotypes. A dendrogram was constructed following Ward's clustering approach using the 80 shortlisted rice germplasms for their genetic relatedness among the genotypes (Figure 3). The shortlisted germplasm lines were clearly grouped into various groups and subgroups based on 9 trait descriptors (Figure 3). The cluster analysis using Ward's method showed two major clusters in which cluster I accommodated eight genotypes only and second one included rest 72 genotypes. The tolerant genotypes Kanchan, Jagannath, Manika, Lahangalata, Dimapur and Mahalaxmi along with two moderately tolerant genotypes Sreebalaram and Mahipal were grouped together in one distinct sub cluster

Genetic structure analysis

The results of the population structure analysis is much useful to the plant breeders for enhancement of Fe-toxicity tolerance in the developed breeding materials. In this investigation, the analysis obtained by analyzing in STRUCTURE software categorized the studied population into 3 classes. This is inferred from the graph generated by taking K and ΔK values showed a peak at K=3 (Figure 4a). Overall proportion of membership in each of the cluster was 0.325, 0.289 and 0.386 in the subpopulation 1, subpopulation 2 and subpopulation 3, respectively. The three subpopulations showed fixation index (Fst) values of 0.3836, 0.2848 and 0.2102 for population 1, population 2 and population 3, respectively. The allele-frequency divergence among the subpopulations based on point estimates of net nucleotide distance computed varied from 0.1116 to 0.1576. Average distances (expected heterozygosity) between individuals in the subpopulation 1, subpopulation 2 and subpopulation 3 were 0.3416, 0.3604 and 0.4100, respectively. The structure analysis at peak value of ΔK at K=3 for the genotypes clearly differentiated population into low, medium and high Fe-toxicity tolerance germplasm lines (Figure 4b). Therefore, the peak of ΔK at K=3 was taken for further analysis of the results. The shortlisted population was classified into three structure groups. The population in the panel was found to show a relationship with the Fe-toxicity tolerance of genotypes present in the panel (Figure 4b). The subpopulation 1 consisted the Fe-toxicity tolerant genotypes, whereas subpopulation 3 consisted tolerant genotypes along with seven moderately tolerant and two susceptible ones. But Subpopulation 2 consisted all susceptible genotypes except Hatipanjara and Latamahu and majority of the moderately tolerant genotypes. The alpha value estimated by the software was 0. 1955 at K=3. A leptokurtic distribution curve was observed for alpha-value and for 3 subpopulations at K=3 (Figure 5).

Marker-trait association

Marker-trait association study revealed significant association of markers with Fe-toxicity tolerance in the studied rice genotypes. The markers significantly associated using both GLM and MLM model at $p<0.05$ with different parameters taken under Fe toxicity stress are presented in Table 3. The r^2 value at $p<0.05$ ranged from 0.059 to 0.23 and 0.051 to 0.157 with GLM and MLM model, respectively. Eighty one marker trait combinations were obtained with GLM and/or MLM models of which 24 combinations were with both models. Considering both GLM and MLM at $p<0.05$, 5, 3, 1, 3, 3, 4, 3 and two markers were associated with DFF, plant height, grain number, seed test weight, grain yield, tiller number, Fe content in grain and LBI, respectively under Fe toxicity stress. The LBI, a significant parameter for Fe toxicity tolerance, was associated with markers RM5897 and RM206 by using both GLM and MLM models with around 10% and 7.5% phenotypic variance at $p<0.05$, respectively. RM488 and RM5638 were also associated with LBI by using GLM model only. The QQ plot showed all the traits under study were significantly associated with the markers (Figure 6).

All the markers associated with DFF, plant height, grain number, test seed weight, yield, tiller number, Fe content in grain and LBI under Fe toxicity stress were distributed in all 11 chromosomes except chromosome 12. The two markers RM5897 and RM206 associated with LBI were located on chromosome 2 and 11 at 6.7 and 22.01Mb positions, respectively.

Discussion

Majority of the high yielding rice varieties are not tolerant to Fe-toxicity stress. There is a need to breed tolerant high yielding rice varieties for increasing production from the affected region of the country. Identification of robust markers for the trait is essential for incorporation of tolerance through MAS breeding. In our phenotyping results, the 80 shortlisted genotypes showed wide variation for leaf bronzing scores staring from 1 to 7 SES score. Three clear phenotypic classes were obtained for iron toxicity tolerance in the studied population. Fortunately, the shortlisted population worked well for association and structure analysis and seems to be appropriate as revealed from structure analysis. The principal component analysis placed the studied shortlisted genotypes as per their LBI and other traits and distributed into different spots in the four quadrants (Figure 2). Besides, the Wards clustering also differentiated into many clusters and sub cluster (Figure 3). Therefore, it is concluded that the shortlisted germplasm lines possess considerable genetic variation for iron toxicity tolerance and effective panel for studying marker-trait association. Earlier reports on existence of natural variation for iron toxicity tolerance in the rice germplasm were also published by many researchers [22,24-28].

Evaluation of 80 genotypes under stress condition revealed that few genotypes produced better yield under the stress condition. This provided the clue for breeding of Fe-toxicity tolerance along with high grain yield in rice is possible. Similar way, the relationship of high grain yield and protein content in rice was reported earlier [38]. From the genotype-trait biplot analysis, the placement of promising genotypes in the encircled area accommodate the high yielding and Fe-toxicity tolerant genotypes together (Figure 2). Therefore, improvement of Fe-toxicity tolerance along with high grain yield is possible in rice.

The various groups and sub-groups obtained from Ward's clustering and placement of genotypes in the PCA quadrant provided clue about involvement of different genes/QTLs responsible for different classes (Figure 2&3). These groups in the population indicated the presence of linkage disequilibrium in the population and provided scope for association of Fe-toxicity tolerance in the population. Similar type of experimental results on marker-phenotypic association were reported earlier for different phenotypic traits in rice [33,38,41,42,45-51]. A high level of genetic diversity was detected in the shortlisted population. The present investigation on genetic diversity is almost similar to the earlier findings showing high genetic diversity parameters for single trait [52-55]. However, many earlier reports also detected moderate diversity parameters for various traits in many rice populations [38,56-59].

Population structure is required to know the different groups of individuals present in a genetic makeup of individuals present within a population. The crop improvement genetic gain will be rapid by utilizing germplasm lines with known genetic makeup from a population. The shortlisted genotypes were grouped into 3 structure groups. The population was divided into subpopulations based on Fe-toxicity tolerance (Figure 4b). The red and blue bar inferred ancestry genotypes (subpopulation 1 and 3) were mainly associated with moderate to high tolerance to the stress. Majority of the green bar groups (2nd ^{t} subpopulation) were with moderate to low in tolerance to Fe-toxicity tolerance. Thus, structure analysis at the 1st peak at K=3 categorized the population into 3 subgroups as per the tolerance level. We detected low alpha value (α=0.1955) for the tolerance indicating a common primary ancestor for F-toxicity tolerance. Subsequently, the evolution of subpopulations with admix genotype might have occurred through natural hybridization and development of many admix genotypes. The inferred ancestry obtained from structure analysis indicated the clues for QTLs responsible for small effects. These small effects QTLs may be pooled together in a single background through molecular breeding. Earlier publications on association studies also provided similar opinion on QTLs/gene(s) stacking for enhancement of the trait [38,41,42,54].

The subpopulations were different from each other as per their F_{ST} values and their distribution pattern at K=3 indicating that the populations are different from each other. Also, it is clear that the within and between F_{ST} values of the shortlisted lines were different and hence, genetic differences among the subpopulations exist. It is expected that parents selected from population possessing higher Fst values, there is better recovery of progenies with Fe-toxicity tolerance in recombination breeding. Therefore, efforts need to be given to pyramid the QTLs controlling iron toxicity tolerance from different populations resulting in higher tolerance in the progenies. Similar type of opinion were also reported by earlier workers for increasing grain protein content, high and low temperature stress tolerance and grain yield in rice [38,41,42,60].

The two significantly associated markers RM5897 and RM206 for Fe toxicity tolerance in terms of LBI detected through both GLM and MLM models with r^2>0.05 and high F value indicated a very strong association. Earlier reports showed that three regions on chromosome 2 (25.86-26.66Mb, 31.49-35.13Mb and 2.76Mb) to be associated with Fe toxicity tolerance in terms of LBI [24,61,62]. But RM5897 is located at 6.733Mb position, hence, may be a novel QTL controlling Fe- toxicity and named as *qFeTox2.1*. The marker RM5897 is also showed association with grain-Fe content, which is corroborated with the results of [26,62,63]. These independent reports showed that this region of 2.88Mb to 10.3Mb of chromosome 2 has multiple QTLs responsible for Fe-content in rice grain. Hence, this region can be said as hot spot for Fe content in rice grain, also evident from our result. These QTLs are validated in this study. It also indicates that these QTLs for grain Fe content are co-localized with Fe toxicity tolerance QTL. So, it may be inferred that these two traits grain Fe content and Fe toxicity tolerance may share some common pathway for channelization of Fe. This needs more detailed study for confirmation.

RM206 located on chromosome 11 at 22.015Mb position was associated with LBI using both GLM and MLM model with phenotypic variance around 9%. This region of chromosome 11 has been reported to govern various parameters under Fe toxicity stress. [61] reported *qFeRSL11* at 20.86Mb position, whereas *qSwc11*and *qSfw11* (23.03-23.95Mb) were reported by [64]. Also, [62] showed regions from 19.56 to 28.28Mb is responsible for Fe toxicity tolerance. Hence, this QTL is validated for Fe toxicity tolerance.

Also, two other markers, RM105 and RM1278 located on chromosome 9 and 11, respectively were significantly associated with grain Fe content showing phenotypic variance of around 7% each. The RM105 region is reported for grain Fe content QTL *qFe9.1* by [65]. Hence, this QTL has been validated in the present study. RM1278 located at 4.56Mb position is co-localized with Fe toxicity tolerance (LBI score) reported by [24].

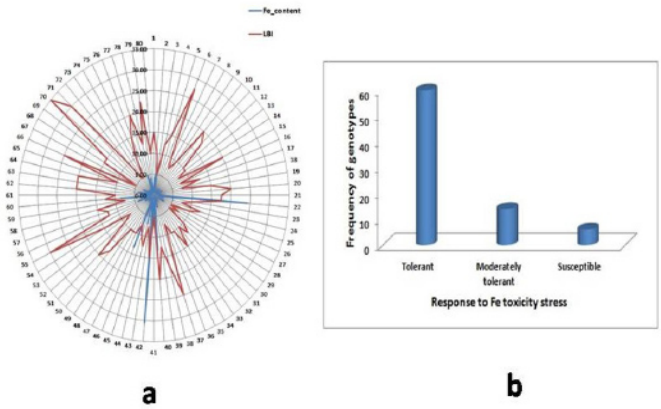

Figure 1: (a) Radar graph showing Leaf bronzing index and grain Fe content and (b) frequency distribution for response to leaf browning index in 80 shortlisted studied rice landraces and varieties

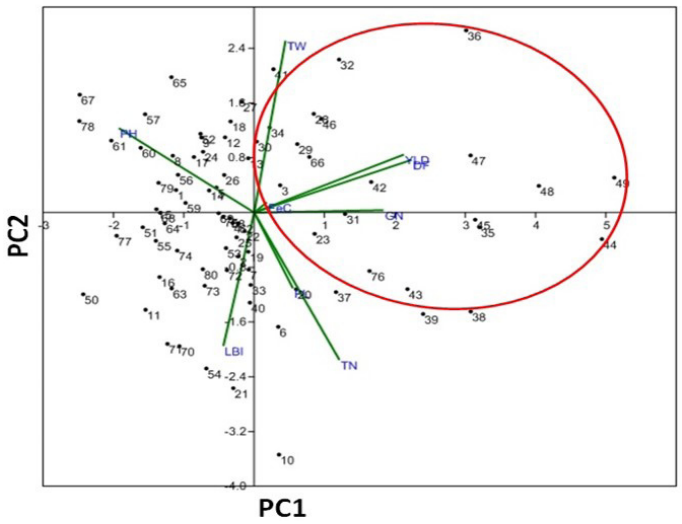

Figure 2: The biplot graph generated using 9 traits of 80 shortlisted germplasm lines in two main principal components. LBI: Leaf bronzing index; DFF: days to 50% flowering; Fe-C: Iron content in grain. yld: grain number; GN: number of grains/panicle; PH: Plant height (cm); TN: tiller number; TW: seed test weight (g); grain yield (kg/ha). The spot in the biplot denotes the genotypes serial number as enlisted in Table 1.

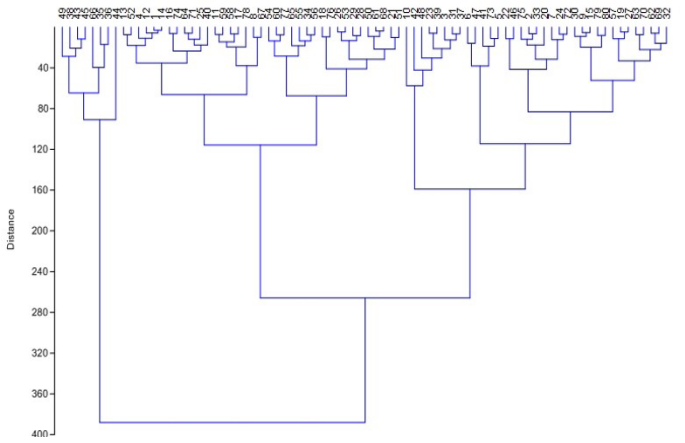

Figure 3: Ward's Cluster diagram of 80 shortlisted genotypes based on nine morphological descriptors

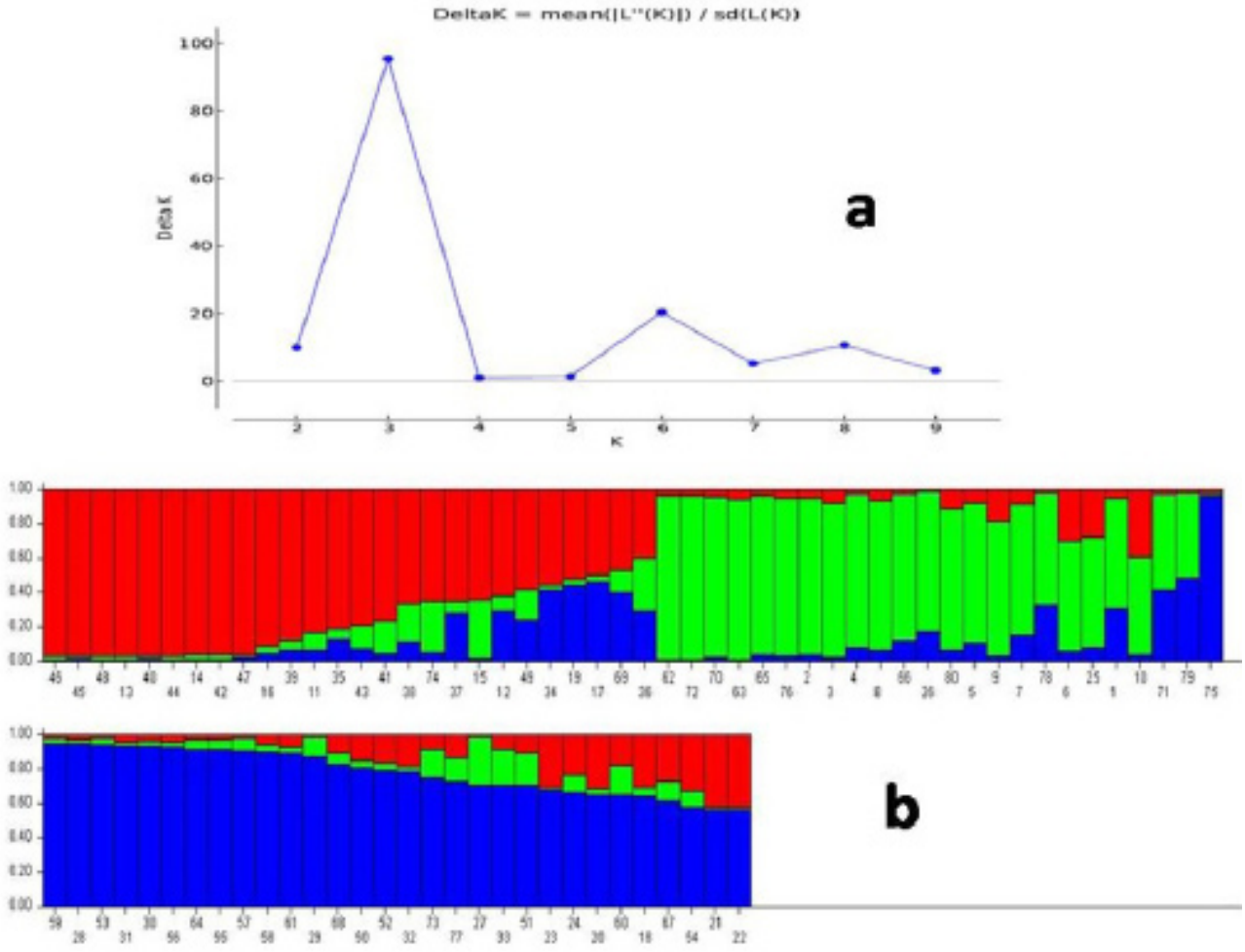

Figure 4: (a) Graph generated by ploting delta K Vrs. K for determination of peak value and (b) the genetic structure groups obtained for the studied panel population and sorted as per the group

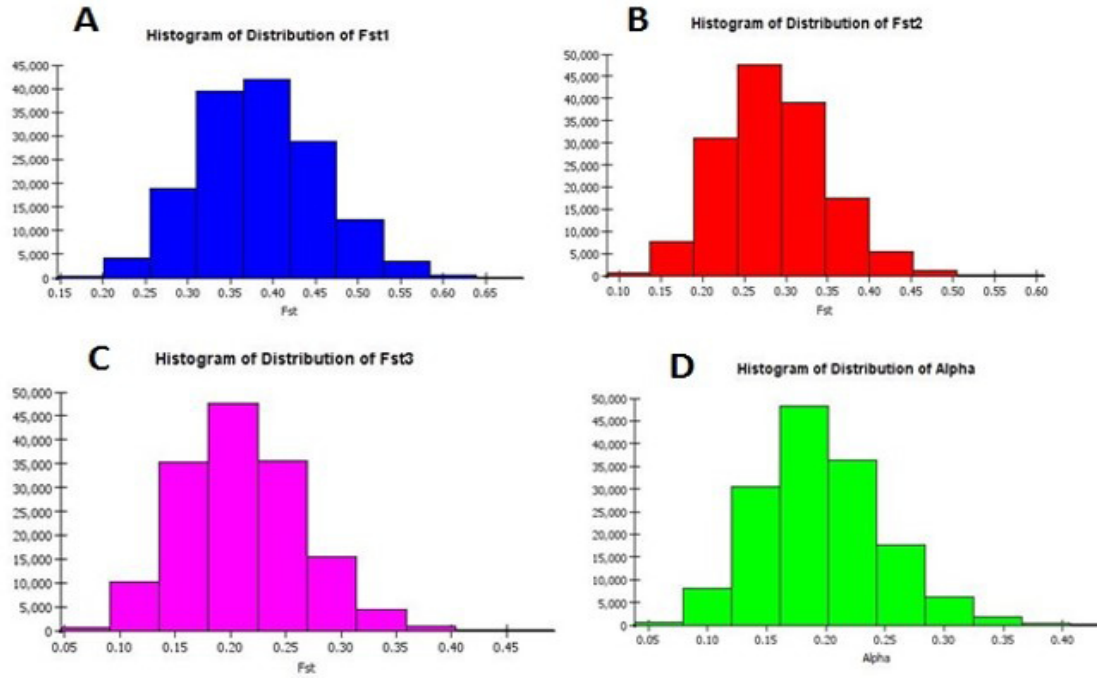

Figure 5: Distribution of Fst values obtained for (a) subpopulation 1; (b) subpopulation 2; (c) subpopulation 3 and (d) alpha value

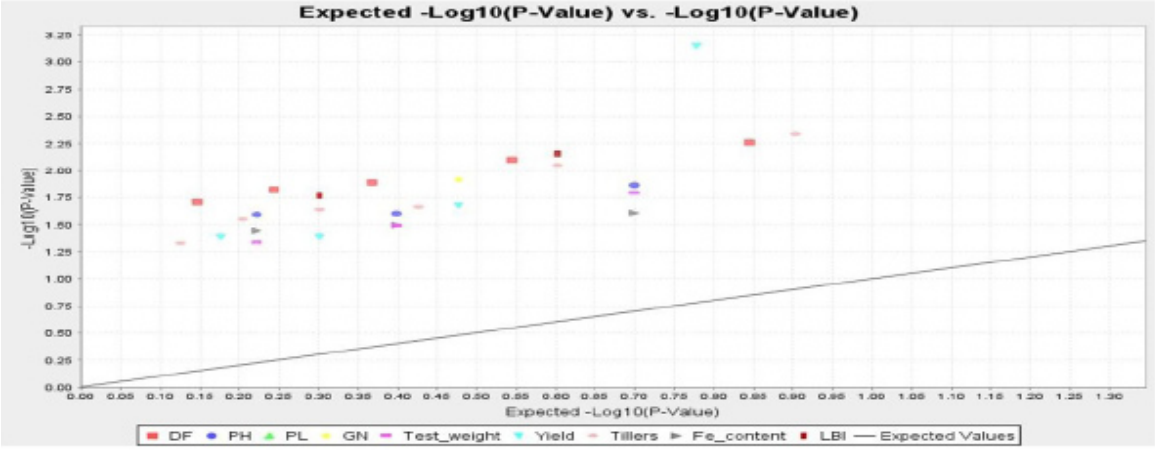

Figure 6: Quantile-Quantile plot showing the significantly associated molecular markers with the traits.

Table 1: Mean Leaf bronzing, Fe-content, yield and morphologic traits of the shortlisted genotypes under Fe-toxicity stress during wet season, 2017 and 2018.

Sl.No.	Genotypes	Days to 50% flow.	Plant height (cm.)	Panicle Length (cm.)	Grains/ panicle	Seed test weight (g.)	Panicles /plant	Grain-Fe content	LBI	Yield (kg/ ha)	Response to Fe-toxicity
1	Agnisar	103.50	126.55	25.32	98.10	21.30	4.75	1.09	3.00	21.15	R
2	Malata	106.00	116.65	24.88	116.80	12.40	5.14	4.26	2.00	24.02	R
3	Kabir	106.50	92.94	21.85	121.15	17.51	5.50	6.03	1.00	21.55	R
4	Nadalghanta	104.00	129.51	24.83	107.80	20.45	7.00	0.82	2.50	24.48	R
5	Latachaunri	104.50	133.17	23.31	169.16	18.15	4.72	1.58	3.00	21.30	R
6	Nalikamala	103.50	109.99	27.81	167.50	20.86	6.30	0.75	5.50	21.67	S
7	Sarubhajana	102.00	104.75	21.46	133.80	17.95	8.01	0.78	2.00	19.60	R
8	Luna	106.00	163.00	27.18	90.10	17.60	5.20	1.75	1.50	25.65	R
9	Abhiram	103.50	141.91	25.35	117.80	22.95	5.14	1.00	2.00	24.88	R

10	Sebati	99.50	61.80	22.75	103.50	14.83	9.08	0.85	7.00	20.10	S
11	Ahirman	101.50	136.25	26.36	88.10	15.90	6.97	0.91	3.50	19.56	R
12	Bhutmundi	106.00	127.73	24.03	100.50	23.34	5.33	2.13	2.00	24.14	R
13	Makarkanda	108.00	120.40	20.68	100.10	23.17	6.97	0.63	2.00	22.07	R
14	Jata	105.50	128.02	25.35	95.80	21.32	6.39	0.64	1.50	19.51	R
15	Khajurkandi	102.50	138.45	21.95	116.00	21.38	6.07	2.28	4.00	20.19	MR
16	Tulasimali	103.50	126.58	25.48	96.10	11.30	5.73	1.34	1.50	17.51	R
17	Nalibaunsagaja	104.00	136.75	22.50	92.80	26.38	7.12	0.78	2.50	21.77	R
18	Malabati	102.50	105.23	23.33	81.16	25.82	5.07	1.23	1.50	26.18	R
19	Pateni	107.00	138.05	25.50	141.85	17.65	6.97	1.84	3.00	21.58	R
20	Nikipakhia	110.50	107.05	24.76	110.25	14.23	5.99	1.55	4.00	25.28	MR
21	Malliphujajhuli	104.50	104.55	26.85	90.55	14.92	8.51	1.16	3.50	16.43	MR
22	Jhilli	103.50	115.40	23.01	123.85	18.57	6.06	24.34	3.50	23.49	MR
23	Bharati	110.50	102.34	23.38	126.75	14.83	6.03	1.30	2.00	23.74	R
24	Hunder	104.00	118.05	20.55	128.55	17.47	4.75	2.18	1.50	21.46	R
25	Sapri	109.00	142.05	22.47	101.25	15.35	7.72	2.30	2.50	23.93	R
26	Dholabankoi	108.00	117.61	23.05	86.55	19.20	5.39	2.19	2.00	21.92	R
27	Korkaili	107.00	141.32	23.10	142.25	20.77	5.09	2.89	1.00	24.76	R
28	Kalamulia	116.00	117.68	23.33	86.25	20.86	5.08	0.71	1.50	26.27	R
29	Kusumkunda	110.50	111.56	21.28	93.16	23.19	7.22	0.91	1.00	23.52	R
30	Saraswati	110.00	138.89	19.70	119.16	21.53	7.25	0.64	2.00	23.93	R
31	Budhamanda	110.00	96.28	20.10	110.16	25.43	9.05	0.78	2.50	23.12	R
32	Khajara	115.00	124.61	21.26	139.24	33.00	6.92	0.62	2.00	21.24	R
33	Matiakhoja	108.00	123.53	24.41	112.30	11.70	6.22	1.60	3.00	24.82	R
34	Haribhohg	108.00	126.48	23.70	71.83	25.54	6.44	1.06	2.00	29.73	R
35	Lahangalata	112.00	98.13	23.05	226.10	18.82	7.88	1.11	2.00	30.22	R
36	Dimapur	112.00	95.63	26.26	211.16	37.06	5.05	1.42	1.50	28.46	R
37	Padmakesari	109.00	96.13	25.08	115.50	22.15	7.17	2.92	5.00	26.06	R
38	Sreebalaram	110.00	73.55	23.19	178.80	23.80	9.84	0.00	3.50	26.33	MR
39	Dhanashree	112.50	98.95	27.84	126.60	17.65	8.83	1.88	2.50	28.49	R
40	Khndiratnachudi	106.00	132.32	23.51	101.30	13.60	7.78	13.23	4.00	27.35	MR
41	Ruksal	109.00	140.74	22.60	156.30	22.60	4.83	1.85	1.00	26.02	R
42	Harisankar	114.50	97.29	23.80	111.00	25.64	7.00	30.48	3.00	21.12	R
43	Jagannath	108.50	89.29	23.00	187.00	15.40	8.47	0.97	1.50	24.42	R
44	Mahalaxmi	117.00	77.79	22.69	261.33	18.05	7.92	7.05	2.50	42.11	R
45	Manika	111.00	84.22	27.07	198.60	18.77	5.70	0.86	2.00	32.94	R
46	Urbashi	108.50	122.30	23.88	124.66	21.53	5.44	13.41	1.50	31.06	R
47	Rambha	114.00	108.12	27.27	157.00	22.93	6.19	0.86	2.00	38.09	R
48	Salivahan	122.00	87.50	24.27	131.00	17.84	6.78	0.77	2.00	38.68	R
49	Kanchan	120.50	88.30	22.90	179.42	19.30	7.92	0.60	2.00	45.28	R
50	Nini	97.00	120.28	23.84	91.80	22.19	7.23	1.85	3.50	8.43	MR

51	Jubaraj	105.00	107.17	21.87	81.50	23.06	5.48	0.62	4.00	11.43	MR
52	Champa	105.00	119.42	20.30	109.50	24.35	5.84	0.89	3.00	19.05	R
53	Veleri	108.00	114.65	23.95	88.20	15.15	6.73	1.55	3.00	17.94	R
54	Hatipanjara	103.50	132.68	21.50	68.10	12.45	10.75	2.47	3.00	22.81	S
55	Latamahu	103.50	115.85	19.78	72.10	21.18	5.44	0.99	6.00	25.43	S
56	Dhusura	103.00	126.01	25.77	65.10	23.41	5.33	1.09	2.50	23.74	R
57	Sagiri	101.50	142.90	22.80	131.80	20.11	3.33	4.26	2.50	23.34	R
58	Bayabhanda	108.50	134.40	25.20	93.80	18.94	6.26	1.24	3.00	23.49	R
59	Dhabalabhuta	104.00	128.90	23.37	85.10	16.09	5.99	1.03	1.50	22.88	R
60	Bangali	101.50	137.00	26.57	75.50	23.01	4.16	8.52	2.50	22.60	R
61	Mugei	100.00	117.40	20.38	92.80	22.92	4.62	0.79	2.00	15.44	R
62	GeleiB	105.50	127.72	22.47	139.80	16.68	5.33	1.24	4.00	26.58	MR
63	Juiphula	102.50	126.80	21.55	144.80	13.41	6.10	4.06	4.00	17.69	MR
64	Madia	106.00	129.20	22.85	100.50	18.35	5.14	1.00	4.00	18.83	MR
65	Nilarpati	104.00	126.25	19.65	82.10	26.69	5.04	1.99	2.00	22.72	R
66	Mahipal	106.00	124.50	21.75	186.00	23.00	4.50	0.58	5.00	32.91	MR
67	Banda	106.00	160.40	21.35	81.00	16.48	3.89	3.00	3.00	17.17	R
68	Kakiri	103.00	118.16	21.25	89.50	21.25	6.28	1.24	3.00	17.17	R
69	Chudi	106.00	127.35	24.76	140.50	19.44	5.39	0.53	3.00	18.99	R
70	Umarcudi	105.00	120.80	26.91	140.50	15.60	4.34	1.70	7.00	17.38	S
71	Champeisiali	108.00	135.31	25.06	99.25	15.56	6.64	0.64	6.00	15.96	S
72	Ratanmali	104.00	111.15	25.00	125.50	14.92	6.19	0.59	2.00	18.68	R
73	Anu	101.00	125.15	24.75	159.25	12.39	6.95	0.70	3.00	16.55	R
74	Karpurakranti	103.50	124.90	23.82	104.55	11.47	5.99	1.52	3.00	18.83	R
75	Dhinkisiali	109.00	120.50	23.15	117.50	17.72	6.22	1.20	2.00	15.44	R
76	Sunapani	107.50	100.90	26.60	83.85	20.24	7.97	0.85	3.00	35.28	R
77	Jalpaya	102.00	130.03	23.37	74.10	17.84	5.02	4.80	4.00	19.42	MR
78	Mayurkantha	99.50	146.85	20.50	91.85	20.76	4.25	0.80	2.50	20.96	R
79	Nalijagannath	105.00	128.50	19.02	125.25	21.48	5.16	4.14	4.50	17.63	MR
80	Ramakrushnabi-lash	104.50	131.80	21.06	125.25	12.17	7.56	0.66	3.00	20.07	R
$LSD_{5\%}$		11.32	13.51	2.84	13.74	1.65	0.69	0.51	-	3.17	
CV%		3.12	5.63	6.52	12.13	8.23	10.35	6.35	-	11.86	

Table 2: The list of primers and molecular diversity parameters estimated from 80 genotypes using the SSR markers.

Sl.No.	Marker	No. of alleles	Minimum size of allele	Maximum size of allele	Major allele frequency	Gene diversity	Hetero zygocity	PIC value
1	RM452	3.0000	240	265	0.7097	0.4209	0.0323	0.3449
2	RM471	4.0000	130	135	0.7260	0.4319	0.0685	0.3882
3	RM3	4.0000	110	150	0.8200	0.3124	0.2000	0.2920
4	RM31	3.0000	90	110	0.5985	0.5605	0.0455	0.4990
5	RM237	3.0000	130	150	0.6761	0.4785	0.0282	0.4187
6	RM407	2.0000	160	180	0.7313	0.3930	0.0597	0.3158

7	RM590	3.0000	130	150	0.4800	0.6194	0.0267	0.5429
8	RM105	3.0000	100	131	0.6985	0.4666	0.2794	0.4212
9	RM278	3.0000	135	140	0.5071	0.6211	0.0429	0.5512
10	RM168	2.0000	115	150	0.9219	0.1440	0.0313	0.1337
11	RM3331	3.0000	90	175	0.6892	0.4766	0.0811	0.4295
12	RM5638	4.0000	200	210	0.4420	0.6293	0.1594	0.5526
13	RM202	3.0000	160	250	0.4918	0.6242	0.3770	0.5516
14	RM8044	3.0000	200	280	0.4930	0.5888	0.1690	0.5031
15	RM23	2.0000	140	300	0.5379	0.4971	0.0152	0.3736
16	RM5897	3.0000	140	150	0.6267	0.5376	0.0000	0.4804
17	RM232	3.0000	150	160	0.7222	0.4095	0.0476	0.3377
18	RM2416	2.0000	180	250	0.5778	0.4879	0.0000	0.3689
19	RM307	3.0000	120	270	0.8681	0.2349	0.0694	0.2173
20	RM440	3.0000	150	200	0.8861	0.2055	0.2025	0.1908
21	RM585	3.0000	130	150	0.4844	0.6246	0.0156	0.5508
22	RM432	2.0000	180	190	0.6090	0.4762	0.0128	0.3628
23	RM152	3.0000	140	160	0.5000	0.5444	0.7887	0.4407
24	RM556	1.0000	180	180	1.0000	0.0000	0.0000	0.0000
25	RM205	2.0000	130	170	0.9810	0.0373	0.0127	0.0366
26	RM269	4.0000	180	310	0.3000	0.7352	0.8400	0.6855
27	RM206	4.0000	140	170	0.2759	0.7449	0.1724	0.6972
28	RM309	3.0000	180	210	0.7848	0.3608	0.1013	0.3320
29	RM488	3.0000	150	200	0.5000	0.5678	0.1912	0.4744
30	RM243	4.0000	120	240	0.6908	0.4623	0.0263	0.4041
	Mean	2.9333			0.6443	0.4564	0.1366	0.3966

Table 3: Association of leaf bronzing index, grain Fe-content, grain yield and related traits through MLM and MLM in rice.

GLM analysis				MLM analysis			
Trait	Marker	marker_F	marker_p	Marker R^2	F	P	Marker R^2
DF	RM3	7.82515	0.00649	0.09118	5.68386	0.01955	0.07195
DF	RM407	6.69148	0.01155	0.07901	8.1679	0.00547	0.10339
DF	RM278	8.01619	0.00589	0.09319	7.41202	0.00799	0.09382
DF	RM152	6.09362	0.01575	0.07246	6.18564	0.01501	0.0783
DF	RM448	16.06028	1.39E-04	0.17074	6.47436	0.01292	0.08195
PH	RM3	6.17578	0.01509	0.07337	6.3626	0.01369	0.08054
PH	RM232	9.29464	0.00314	0.10647	5.23065	0.0249	0.06621
PH	RM585	8.90977	0.00379	0.10252	5.18548	0.02552	0.06564
GN	RM5897	23.63845	5.91E-06	0.23257	6.59644	0.01213	0.0835
Test_weight	RM105	6.50236	0.01273	0.07695	4.76309	0.03209	0.06029
Test_weight	RM8044	6.91253	0.01031	0.08141	6.06077	0.01603	0.07672
Test_weight	RM2416	4.07241	0.04703	0.04962	4.11814	0.04584	0.05213
Yield	RM590	4.96152	0.0288	0.05981	5.53593	0.02115	0.07008

Yield	RM307	7.07825	0.00947	0.0832	4.3314	0.0407	0.05483
Yield	RM488	12.70405	6.27E-04	0.14006	12.42326	7.13E-04	0.15726
Tillers	RM 31	6.6193	0.01199	0.07822	4.07704	0.04691	0.05161
Tillers	RM440	8.34221	0.00501	0.09662	8.51	0.00461	0.10772
Tillers	RM152	15.84035	1.53E-04	0.1688	7.18383	0.00897	0.09093
Tillers	RM205	4.89465	0.02987	0.05905	5.00282	0.02816	0.06333
Fe_content	RM 105	6.46279	0.013	0.07652	5.24669	0.02469	0.06641
Fe_content	RM 278	6.70237	0.01148	0.07913	4.56284	0.03581	0.05776
Fe_content	RM5897	7.75066	0.00673	0.09039	4.77469	0.03188	0.06044
LBI	RM5897	9.38405	0.003	0.10739	7.67203	0.00701	0.09711
LBI	RM206	6.61947	0.01198	0.07823	5.95085	0.01698	0.07533

Conclusion

A moderate level of genetic diversity was estimated from the studied population. Among the shortlisted genotypes, only two varieties found to produce grain yield of >4 t/ha consistently were Kanchan and Mahalaxmi. The two lines also showed tolerance to the stress evaluated under Fe-toxicity sick plot. STRUCTURE software grouped all the genotypes into 3 genetic structure groups. LBI showed significant associations with RM5897 and RM 206 by both the models. A novel QTL controlling Fe-toxicity was detected and named as *qFeTox2.1.* Marker RM 206 associated with QTL for Fe-tolerance on chromosome 12 is also validated. GML and MLM detected association of grain-Fe content with markers RM105, RM1278 and RM5897. Chromosome 2 region from 2.88Mb to 10.3Mb showed multiple QTLs responsible for Fe-content and hot spot for Fe content in rice grain. QTL *qFe9.1,* controlling the grain Fe-content and co-localization of RM1278 with Fe toxicity tolerance are validated in the present study. Tillers/plant showed association with RM31, RM440, RM152 and RM205 by both the models. Grain number is strongly associated with marker RM5897 detected by both the models. Grain yield/plant was detected to be associated with markers RM5908, RM307 and RM488.

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Declarations

Ethical Standards

Conflict of Interest: The authors declare that they have no conflict of interest.

We declare that this research involved no human participants and/or animals

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Data availability

All data generated or analyzed during this study are included in this published article

Author contributions

SK Pradhan, IC Mohanty and E Pandit contributed to the study conception and design. Material preparation, data collection and analysis were performed by Arjun P, M Wagh, S.Pawar and J Meher. The first draft of the manuscript was written by SK Pradhan & E Pandit. All authors read and approved the final manuscript.

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