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Research Article

Multiple interval mapping of QTLs and epistasis for iron toxicity tolerance in segregating population of *Indica* rice

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Abstract

The global average temperature has increased by approximately 0.5 °C, over a last few decades and is projected to continue to increase. Environmental stress factors such as, elevated temperature, salinity, toxic elements (Fe, Al, Cd, Cr, Pb, Zn and As), drought and rising CO_2 affect plant growth and make a growing threat to agriculture. Rice is a primary food crop in the world and the establishment of rice crop in acidic soil and in marginal soil is a major goal for the improvement of rice production to fulfill the food security. Among environmental stresses, Fe^{2+} toxicity is one of the main stresses in limiting the cereal crops production. Tolerant rice genotypes that can tolerate the high concentration of Fe^{2+} toxicity are the potential source genes for rice tolerance improvement in Fe^{2+} toxicity. In this research work, the genetic basis of seed germination traits and growth traits was investigated in rice using (multiple interval mapping) MIM. Many rice genotypes serve as source of tolerant against toxic metal ion like Fe^{2+}, could be an important factor in controlling the sever effect of Fe^{2+} toxicity on germination and seedling growth traits. The F_3 progenies of cross between Fe^{2+} toxicity tolerant cultivar 'Pokkali' and susceptible cultivar 'Pak basmati' were test against the optimized level of Fe^{2+} toxicity at germination, to determine the mode of inheritance to Fe^{2+} toxicity tolerance. Wide range of continues variation was found in F_3 progenies. Among the 49 quantitative germination trait and 23 growth trait loci (QTLs) on chromosomes 1, 2, 4, 6, 8 and 9 linked with tolerance to Fe^{2+} toxicity was mapped. Additionally, 21 QTLs for germination traits and 9 QTLs for growth traits were classified as major QTLs using MIM. For germination and growth traits, notable epistasis between the chromosome 1, 2, 4, 6 and 11 was detected across germination and growth traits. Our results suggest that the tolerance mechanisms at germination and seedling phases could differ for Fe^{2+} toxicity. QTLs detected in this study for germination and seedling growth could be a source of new alleles for development of tolerance rice to Fe^{2+} toxicity varieties and transformation, gene cloning and gene editing in the future

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Keywords: iron toxicity; multiple interval mapping (MIM); 'Pak Basmati'; 'Pokkali'; QTLs

Introduction

Rapid increase in urbanization and industrialization are significantly contributing to elevate the toxic elements in atmosphere deposits and pollutes the soils that ultimately influence plants directly. It is a rising public concern that agriculture soil has been polluted due to toxic elements (Li *et al*., 2019*)*. Polluted soil associated with loss of productivity and due to rapid growth of population, the food shortage has become a serious problem worldwide (Shah *et al*., 2020). Meanwhile, there are various reason associated with future global food shortage such as reduction of available arable lands due to soil erosion, degradation, unsustainable farming and toxic soils, decline of water for agriculture and the climate change *(*Maximillian *et al*., 2019). Increase of heavy metals in soils is commonly spread and reducing the crop production (Özyiğit *et al.,* 2021). Of all heavy metals, Fe^{2+} is of most concern due to its easily uptake by root and mobilization in plants. Rice is a primary food crop for the world. Approximately 90% of world rice is cultivated in Asia (Rehman *et al*., 2022). Rice is the main crop grown in more than 100 countries worldwide and is harvested in an area of approximately 158 million hectares (Banik *et al*., 2020). The cultivation of rice by direct seeding is a common practice to produce high yields and profitability due to its low inputs and labour demand (Farooq *et al*., 2011). However, farmers have been facing many challenges since the adaptation of direct seeding of rice, such as abiotic stresses, weed incursion, panicle sterility, nutrient availability, pests, and water management. The challenge for rice breeders is to improve rice productivity in limited arable land and cultivation on marginal lands, which is characterized as low productive land due to abiotic stresses (Spiertz, 2013). Among all challenges, abiotic stresses are the major bottleneck in direct seeding culture of rice in arable and marginal lands (Farooq *et al*., 2011). Abiotic stresses, including acidification, heavy metal (loid) toxicities, drought, salinization, and diseases, are the main constraints that inhibit the high production of rice (Shamshuddin *et al*., 2014).

Heavy metal such as Fe^{2+} toxicity severely affect at germination and ultimately cause poor crop establishment, as reported in the tropics (Shamshuddin *et al*., 2014). Iron (Fe) is an essential nutrient for plant metabolism, but it becomes toxic when available in excess amounts to plants (Sharma *et al*., 2022). In aerobic soil, Fe is present in the form of ferric hydroxide and is not available to plants in excess (Tripathi *et al*., 2014*)*. However, in an anaerobic situation, the oxygen supply is reduced and oxygen can only be supplied to respiring soil organisms through the diffusion from the closest aerobic zone (Armstrong and Drew, 2002). Under the low availability of oxygen in saturated soil, the soil biota utilizes the other nutrient compounds that are present in both dissolved and solid forms as electron acceptors to retain their metabolic processes. Soil microbial metabolism is the conversion of oxidized nutrient compounds of soil to reduced forms under anaerobic conditions (Poláková *et al*., 2021) and promotes the reduction of elements such as iron (Fe), manganese (Mn), nitrogen (N) and sulphur (S). The reduction of these elements causes the release of other toxic elements such as aluminium (Al) due to depletion of pH and hazards for rice plants. Reduced Fe^{2+} accumulation can further increase in the soil when a high concentration of salts is likely to compete for binding with soil sites. The increase in Fe^{2+} concentrations in soil solution causes oxidative stress, which leads to metabolic disorders in plant tissues. The severe toxicity of Fe^{2+} at younger stages of rice plants causes complete crop failure, while a yield failure of 15-30% has been reported (Wu *et al*., 2014).

New methods are consequently essential to integrate the agronomically important traits linked with tolerance to abiotic stresses and high yields (Witcombe *et al*., 2008*)*. Rice genotypes differ widely in Fe^{2+} tolerance, which makes it possible to develop rice cultivars with an enhanced Fe^{2+} tolerance genetic background. Genetic tolerance for the detection of QTLs linked to Fe^{2+} toxicity and their incorporation into high-yielding varieties will significantly enhance and stabilize rice productivity in problematic soils. The subcontinent Indica rice 'Pak basmati' has been chosen for its good nutrition quality; however, it is very sensitive to diseases and toxicity stresses and therefore requires large inputs for achieving high yields. Conversely, the tropical Indica rice

'Pokkali' is characterized as tolerant to disease and toxicity stresses but does not possess higher nutritional quality than subcontinent rice. Thus, 'Pokkali' characterizes a significant reservoir of genes that can be induced to 'Pak Basmati' to improve their resistance against biotic and abiotic stresses. The main objective of this study was to identify the major putative QTLs linked to Fe^{2+} toxicity tolerance in germination, seedling growth parameters and epistasis interactions between QTLs using a segregating population derived from the cross 'Pak basmati' ×'Pokkali'.

Materials and Methods

Plant materials

The two varieties of Indica rice 'Pokkali' and 'Pak basmati' was kindly provided by MARDI, RRI Lahore Pakistan and IRRI, contrast for Fe^{2+} toxicity tolerance was selected for QTLs analysis linked Fe^{2+} toxicity tolerance for germination and growth traits. The rice material was $F_{2:3}$ progenies consisting of 129 (Dufey *et al*., 2009; Wu *et al*., 2014*)* families each derived from the cross between rice variety 'Pokkali' exhibit tolerance against Fe^{2+} toxicity as 'Pokkali' retained most of the absorbed Fe^{2+} in the roots and stopped or slows the transport of Fe^{2+} to leaves and flag leaf (Jahan *et al*., 2015). 'Pak Basmati' was classified as a relatively sensitive variety against Fe^{2+} (Jahan *et al*., 2021).

Hydroponic condition and parental line screening

Experiments were performed in a growth chamber with optimal condition i.e., temperature set as 25 to 30 °C with day/night lighting for a photosynthetic activity. After sterilization the rice seeds were germinated. Six genotypes 'Pak Basmati', 'Pokkali', 'Penderas', 'Firat', 'MR211', and 'SK1' were screened for selecting genetically divergent parental line that exhibits enough differences in one or more trait of interest. After surface sterilization, the sterilized and imbedded seeds were then placed in wet Petri dishes for two weeks and then subsequently the germinated seeds were transferred in to 4L tank filled with Yoshida solutions addition of $FeCl_3$ (0, 500 mg and 750 mg/L) at pH 4.0 under the controlled environment (temperature 27 °C for 10 h and 30 °C for 14h: humidity 60%). A 0.1 M citric acid was added to avoid the ferric ion precipitation and increase the pH up to 4.0. Seeds were considered germinated when both the plumule (root) and radical (shoot) were extended to approximately more than 2 mm in the range of citation (Ahmed *et al*., 2022). Germination and growth parameters such as Final Germination Percentage (FG %) Germination Rate (GR) Germination Energy (GE) Germination Peak Value (GPV), Germination Capacity (GC), Germination Index (GI); Germination Value (GV) and growth parameters like root length (RL), shoot length (SL) and dry mass of flag leaf, were recorded.

Hybrid confirmation and construction of mapping population

F_1 seeds were produced from the cross 'Pak basmati' and 'Pokkali'. F_1 well grown seeds were evaluated by microsatellite markers. For the selection of polymorphic SSR markers, ten markers RM14, RM7, RM19, RM421, RM526, RM422, RM520, RM261, RM233B and RM303 were selected to check the polymorphism. Out of ten, six markers were selected as polymorphic markers as shown in Table 1. Whereas four SSR markers (RM7, RM19, RM421, RM526), were selected on the bases of good visualization and location of bands to evaluate the F_1 hybridization. Four polymorphic microsatellite markers distributed in different chromosomes of rice were selected for confirmation of four F_1 hybridity, the information regarding primers was obtained from McCouch *et al*. (2001). True F_1 hybrids were advanced to produced subsequent generations.

Table.1 Details of SSR markers used in F_1 hybrid confirmation

Ch. No	Locus	Repeat type and length	Forward primer	Reverse primer	Size range (bp)
1	RM14	(GA)18	ccgaggagaggagttcgac	gtgccaatttcctcgaaaaa	174 -191
2	RM526	(TAAT)5	cccaagcaatacgtccctag	acctggtcatgacaaggagg	242- 266
3	RM7	(GA)19	ttcgccatgaagtctctcg	cctcccatcatttcgttgtt	168-182
3	RM422	(AG)30	ttcaacctgcatccgctc	ccatccaaatcagcaacagc	350-389
3	RM520	(AG)10	aggagcaagaaaagttcccc	gccaatgtgtgacgcaatag	247-269
4	RM261	C9(CT)8	ctacttctccccttgtgtcg	tgtaccatcgccaaatctcc	122-126
4	RM303	[AC(AT)2-10]	gcatggccaaatattaaagg	ggttggaaatagaagttcggt	143-205
5	RM421	(AGAT)6	agctcaggtgaaacatccac	atccagaatccattgacccc	234-242
5	RM233B	(CT)20	ccaaatgaacctacatgttg	gcattgcagacagctattga	162
12	RM19	(ATC)10	caaaaacagagcagatgac	ctcaagatggacgccaaga	219-252

Phenotypic evaluation

Parental line stress optimization was performed at 0, 10mM, 15mM and 20mM of stress levels Fe^{2+} toxicity at pH 4.0 to obtain the threshold level for F_3 phenotyping. At 20mM germination and growth parameters showed significance response to Fe^{2+} toxicity (Jahan *et al*., 2021). Therefore, 20mM was considered as threshold level for F_3 phenotyping. F_3 seeds were exposed to Fe^{2+} toxicity (2000 mg/L) at pH 4.0. Germination and growth parameters were recorded and analyzed

Phenotyping for germination and seedling growth traits

F_3 progenies were exposed to optimized limits (20mM) of Fe^{2+} toxicity at the germination stage, where both parents showed highly significant germination and growth parameters. Seeds of F_3 progenies were kept at 50 °C for five days to break dormancy and surface sterilized by dipping in 70% (v/v) ethanol for 1 minute and in a 2% (w/v) solution of NaOCl for 1 hour followed by washing 4-5 times with deionized water (Ranawake *et al*., 2014). The concentrations of iron used in this study were identified by parental line optimization, where a concentration of 20mM Fe^{2+} consistently reproduced variations in germination and growth parameters. Surface-sterilized and imbedded seeds were then placed in wet Petri dishes (10 mm in diameter) for two weeks in optimized toxicity limits (20mM) at pH 4.0. Experiments were conducted under control conditions where the light and dark periods were 14 and 10 hours, respectively, and the humidity level was 60%. Seeds were considered germinated when both the plumule (root) and radical (shoot) were extended to approximately more than 2mm (Peralta *et al*., 2001). Phenotyping of F_3 progenies was carried out to estimate the tolerance to Fe^{2+} toxicity, and germination and growth parameters were recorded.

QTL analysis (loci identification linked with quantitative trait)

Screening of polymorphism among parental line ('Pak Basmati' and 'Pokkali') was performed**.** Genomic DNA was isolated from fresh leaf tissue of parental lines and F_2 plants by Ikeda method (Ikeda *et al.*, 2001). Four-hundreds of microsatellite markers were screened to determine the polymorphism among the parental line ('Pak basmati' and 'Pokkali'). The reactions of PCR amplification were performed in 12.5uL containing 2uL of genomic DNA, 1uL (2uM) of each primer, 0.0625uL each dNTP, 0.7uL$MgCl_2$ (50mmol/L), 0.125uL Taq DNA polymerase (5 units), Taq buffer (10xPCR) 1.25uL and deionized water 7.3625uL. Amplification was carried out in a thermal cycler (Biorad Thermal Cycler), in the PCR protocol, the denaturation was 94 °C for 2 min followed by 39 cycles of 94 °C for 30 sec for primer annealing 54 °C for 45 and for primer extension 72 °C for 90 sec, followed by 72 °C for 10 min and a hold (forever) at 4 °C.

The selection microsatellites markers were based on previous literature on the rice genetic map and genome sequence maps (Djedatin *et al*., 2016; Edzesi *et al*., 2016; Gramene, 2014; Thakur *et al*., 2013). F_2

marker data was tested for deviation from the expected 1:2:1 genotype ratio using a chi-squared test *(*Javed *et al*., 2013*)*. The relative standard for accepting or rejecting a hypothesis is $p > 0.05$.

Construction of Linkage map

The genetic map was constructed using Mapmaker/EXP version 3.0 *(*Lander and Botstein, 1989; Lander *et al*., 1987*)*. The distances between SSR markers were presented in centimorgan (cM) using the Kosambi function (Manly *et al*., 2001). The base maps of rice constructed by Temnykh *et al*. (2000) and McCouch *et al*. (2001) were followed for comparison.

The "sequence" "group at a LOD score of ≥ 3.0 and the maximum distance of 70cM" commands were used to detect subsets of loci representative of the 12 linkage groups. The "map" command was used to output the maximum likelihood map for the order of markers specified (Chema and Dick, 2009).

Statistical analysis

Statistical software SPSS was applied for analysis of variance (ANOVA, IBM SPSS Statistics18). Mean and standard error of mean among growth parameters were performed using SPSS.

Results

The main objective of this study was to identify the major putative QTLs linked to Fe^{2+} toxicity tolerance.

Parental line screening

The six genotypes were exposed to a pulse toxicity concentration of 0, 500 mg and 750 mg Fe^{2+} in hydroponics. A significant effect of interaction between varieties and Fe^{2+} stresses was determined by ANOVA indicating a differential response of varieties to iron stress as shown in Table 2. Visible effects linked with high iron concentrations were commonly found and some varieties ('Pak basmati', 'Penderas', and 'Firat') showed several symptoms, these effects were reduction in germination parameters and retardation of seedling growth particularly lack of root elongation (Rout and Shahoo, 2015). A significant reduction in germination and growth parameters was observed with the increase concentration of Fe^{2+} toxicity. A major influence of Fe^{2+} toxicity was observed in 'Penderas' and 'Pak basmati' showing that these genotypes are Fe^{2+} sensitive. Whereas, the least effect in germination and growth parameters was found in *MR211* and 'Pokkali', the result showed that these genotypes are Fe^{2+} tolerant, as indicated in Figure 1 and Table 2. The genotype 'Pokkali' showed strong tolerance and 'Pak Basmati' more susceptible genotypes for Fe^{2+} toxicity among all tested genotypes. Therefore, the population derived from 'Pak basmati' and 'Pokkali' were reflected suitable for QTLs determination.

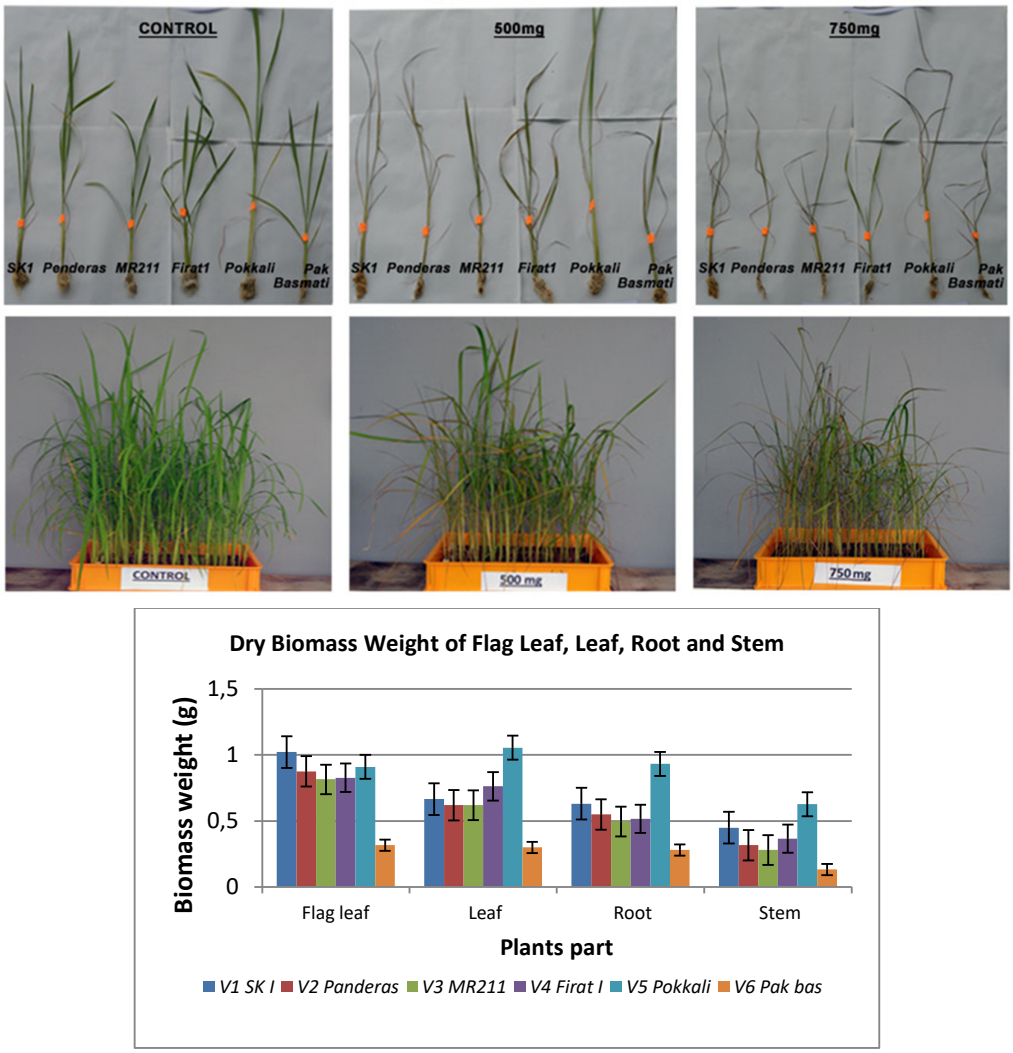


Figure 1. (a) parental line screening indicated significant differences between rice germplasm. Visual comparison between each variety in subsequent treatment, from top, control, 500, 750mg/L and shows that the symptoms of toxicity develop more clearly on the higher concentration of ferrous in a variety (b) represent parental line after 4 weeks treatments, bars represent standard mean of error. As the concentration increase, a significant reduction in plant root, flag leaf and in stem was observed

Table 2. Analysis of variance showing mean sum of squares of germination parameters for six rice varieties at different concentrations (0, 500mg, 750mg at pH 4.0) of Fe^{2+} toxicity

Source of variation	df	Sum of Squares							
		FG%	GR	GE	SG	GPV	GC	GI	GV
Variety	5	130.00ns	0.12ns	43742.22**	354.04**	69.863**	1862.22**	520.00ns	3520.27**
Stress	3	93.33ns	0.11ns	5637.78**	60.88**	12.017**	1077.78**	373.33ns	698.38**
Variety × Stress	20	120.00ns	0.03ns	2602.22**	16.03**	3.149**	1615.56**	480.00ns	177.87**
Error	60	466.67	0.04	7333.333	64.46	12.73	1266.67	1866.67	729.95
Total	90								

**0.01 Significance *0.05 Significance, ns= not significance; FG%=Final Germination Percentage; GR=Germination Rate; GE=Germination Energy; GPV=Germination Peak Value; GC=Germination Capacity; GI=Germination Index; GV=Germination Value.

Hybrid confirmation and construction of mapping population

Four polymorphic microsatellite markers distributed in different chromosomes of rice were selected for confirmation of four F_1 hybridity, the sequence information of primers was obtained from (McCouch *et al*., 2001). A sample of four F_1 plant DNAs as $F_{1\text{-}1}$, $F_{1\text{-}2}$, $F_{1\text{-}3}$, and $F_{1\text{-}4}$ hybridity was confirmed by identifying their heterozygosity at a particular locus representing the alleles of both parents by using selected polymorphic microsatellites markers as shown Figure 2. Three F_1 true hybrid seeds were grown, from which two were grown until maturity while one was not germinated. More than 250 F_2 seeds were harvested. Total 129 F_2 plants produced the required number of F_3seeds for phenotyping analysis (Ocampo *et al*., 2022).

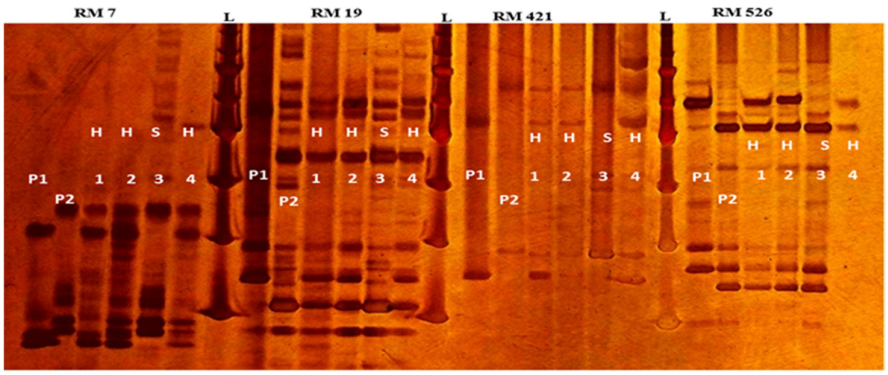


Figure 2. Polymorphic microsatellite marker profiles confirming the F_1 hybridity. P1 = 'Pokkali', P2 = 'Pak basmati', F_1 = P2 × P1 individuals, (1, 2, 3, 4, defines $F_{1\text{-}1}$, $F_{1\text{-}2}$, $F_{1\text{-}3}$, and $F_{1\text{-}4}$), H = hybrid; S= self-pollinated, L = DNA Ladder (Molecular biology, thermos scientific) (100 bp)

Phenotypic performance

Analysis of variance showed the presence of significant genetic diversity between two parents and high phenotypic variations are found between the parents 'Pak basmati' and 'Pokkali'. Therefore, a wide range of genetic variation was observed for all germination and seedling growth parameters except mean germination time, which showed less variation.

A wide range of variation was found for all germination and seedling growth parameters except for MGT in the F_3 progenies, and the range of each trait was greater than the range of the parental line, suggesting transgressive segregation (extreme phenotype). Transgressive segregation determined sufficient genetic variation for QTL mapping (Shang *et al*., 2020). The F_3 progenies have sufficient genetic variation, which is important for QTL analysis linked with tolerance to Fe^{2+} toxicity due to transgressive segregation, as presented in Figure 3 a and 3b.

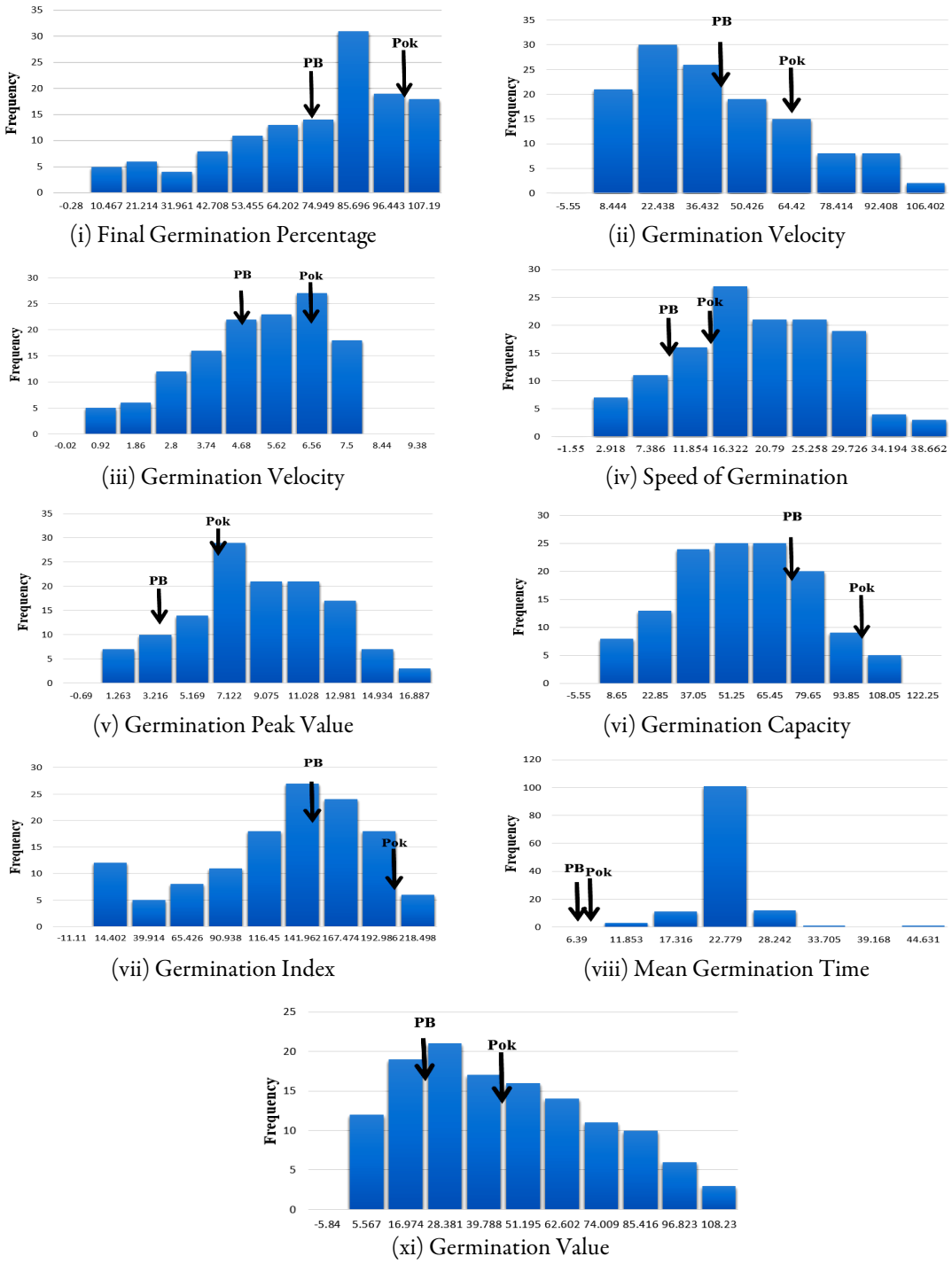


Figure 3 (a). Phenotypic variations and frequency distribution of Fe^{2+} toxicity among $F_{2:3}$ for germination parameters. The arrows in black colour show the parental line mean. PB = 'Pak basmati', Pok = 'Pokkali'

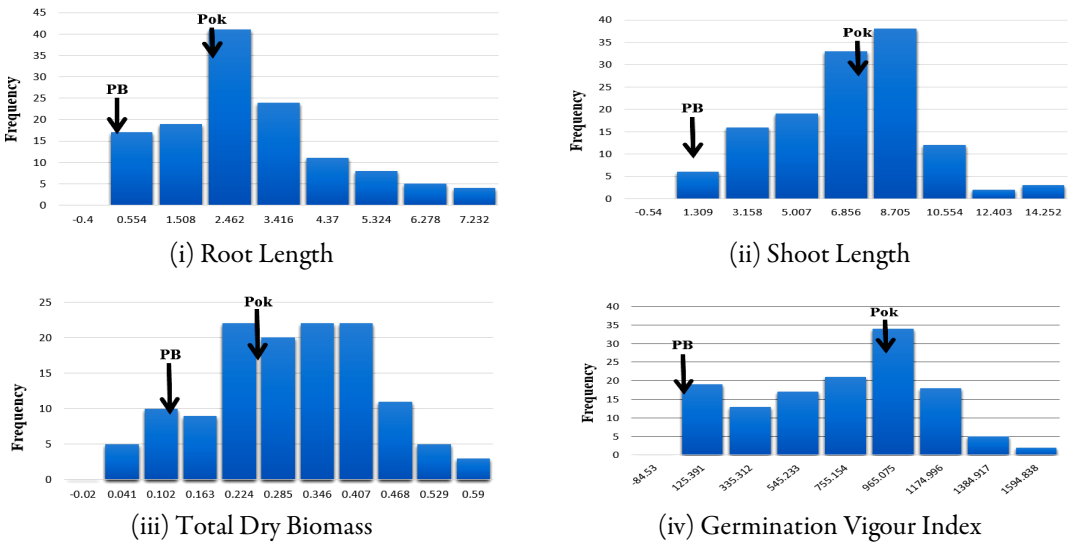


Figure 3 (b). Phenotypic variations and frequency distribution of Fe^{2+} toxicity among $F_{2:3}$ for growth parameters. The arrows in black indicate the parental line mean. PB = 'Pak basmati', Pok = 'Pokkali'

The frequency distribution of germination and growth parameters of F_3 progeny did not exactly fit a normal distribution, conferring use of the Shapiro-Wilk test for frequency normality. However, a wide range of F_3 phenotypic variation distributions suggested that traits are quantitative in nature. For parameters controlled by many genes, parents must simply contain different alleles at various loci, which are then reasserted by recombination in the derived population to produce a wide range of phenotypic values (Shang *et al.*, 2020*)*.

Identifying polymorphic loci between parents

Total 400 randomly SSR markers were selected for spreading all the 12 chromosomes in rice population that used to identify polymorphic loci between the two parents 'Pak basmati' and 'Pokkali'. A 130 SSR markers (32.5%) out of 400 were found polymorphic whereas, 84 markers were selected on bases of clearly high polymorphic. All 129 $F_{2:3.}$ The linkage map had a total map length of ~3435.5cM long. The average interval distance was 7.63cM.

QTL mapping for germination parameter tolerance to Fe^{2+} toxicity

QTL mapping for germination parameter tolerance to Fe^{2+} toxicity is presented in Table 3. Eight QTLs, *QFeFG%1.1*, *QFeFG%1.2*, *QFeFG%1.3*, *QFeFG%1.4*, *QFeFG%2.1*, *QFeFG%4.1*, *QFeFG%6.1* and *QFeFG%6.2*, were detected for trait FG% tolerance to Fe^{2+} toxicity on chromosomes 1, 2, 4 and 6. Seven QTLs, *QFeGE1.1, QFeGE1.2, QFeGE2.2, QFeGE2.3, QFeGE3.1, QFeGE10.1* and *QFeGE12.1,* affecting germination energy (GE) under Fe^{2+} toxicity stress conditions were detected on chromosomes 1, 2, 3, 10 and 12. A total of 10 QTLs, *QFeGVe1.1 QFeGVe1.2, QFeGVe1.3, QFeGVe2.1, QFeGVe3.1, QFeGVe4.1, QFeGVe4.2, QFeGVe6.1, QFeGVe6.2* and *QFeGVe7.1* were detected for the trait germination velocity (GVe) on chromosomes 1, 2, 4, 6 and 7. Three QTLs, *MQFeSG1.1, MQFeSG1.2, MQFeSG4.1* and *MQFeSG6.1,* were detected for the SG region between RM243-RM246, RM128, RM252-335 and RM539-RM454 on chromosomes 1, 4 and 6, whereas an A**×**D epistatic interaction between chromosome 1 (*QFeSG1.2*) and chromosome 4 (*QFeSG6.1)* was detected for trait speed of germination. Four QTLs, *QFeGPV1.1, QFeGPV1.2, QFeGPV4.1* and *QFeGPV6.1,* were detected for the GPV region between RM243-RM246, RM128, RM252-335 and RM528-RM586 on chromosomes 1, 4 and 6 and an A×D epistatic interaction between chromosome 1 (*QFeGPV1.2*) and chromosome 4 (*QFeGPV4.1*) with LOD 1.77 that exhibited 5.3% phenotypic variance

were detected for trait GPV. Four QTLs, *QFeGC1.1 QFeGC1.2, QFeGC4.1* and *QFeGC4.2* detected for germination capacity were located between RM243-RM246, RM128, RM335 and RM335-RM456B on chromosomes 1 and 4. Two A × D epistatic interactions between chromosomes 1, *QFeGC1.1* and *QFeGC1.2* on the same chromosomes and other epistatic interactions were found between chromosomes 1 (*QFeGC1.2*) and 4 (*QFeGC4.2)* to detect trait germination capacity. Seven QTLs, *QFeGI1.1, QFeGI1.2, QFeGI1.3, QFeGI2.1, QFeGI3.1, QFeGI4.1, and QFeGI4.2,* on chromosomes 1, 2, 3 and 4 and an A×D epistatic interaction between chromosome 1 (*QFeGI1.2*) and chromosome 2 (*QFeGI2.1*) were mapped to determine the germination index. Three QTLs, *QFeGV1.1, QFeGV1.2* and *QFeGV6.1,* for germination value were detected on chromosomes 1 and 6. Two QTLs, *QFeMGT1.1* and *QFeMGT6.1,* were analyzed for mean germination time.

QTL analysis of tolerance to Fe^{2+} toxicity for growth parameters

A total of 23 significant putative QTLs linked to Fe^{2+} toxicity tolerance for growth parameters were identified by MIM on 1, 2, 3, 6, 8, 9, 11 and 12, as shown in Table 4. One *MQFeRL1.*1 on chromosome 1 and three QTLs, *QFeRL2.1, QFeRL2.2* and *QFeRL2.3,* on chromosome 2 and three QTLs, *QFeRL3.1, QFeRL4.1* and *QFeRL11,* were detected on chromosomes 3, 4 and 11. Epistasis interactions identified an A×D epistatic interaction between chromosomes 1 and 11 for root length. Eight QTLs, *QFeSL1.1, QFeSL2.1, QFeSL2.2, QFeSL2.3, QFeSL6.1, QFeSL8.1, QFeSL11.1* and *QFeSL12.1,* were detected for shoot length (SL) on chromosomes 1, 2, 6, 8, 11 and 12. Four QTLs, *QFeTDB1.1, QFeTDB6.1, QFeTDB8.1* and *QFeTDB9.1,* for total try biomass (TDB) on chromosomes 1, 6, 8 and 9 were detected. Four QTLs, *QFeGVI1.1, QFeGVI1.2, QFeGVI2.1* and *QFeGVI8.1,* for the germination vigour index on chromosomes 1, 2 and 8 were mapped (Figure 4).

Table 3. QTL analysis by multiple interval mapping for germination parameters

Trait	[a]Chr No	[b]NO of QTLs	Marker interval	[c]Position	[d]Genetic Effect		R2	[e]R2 (%)	[f]VG
					Additive and LOD score	Dominance and LOD Score			
FG%	1	*MQFeFG%1.1*	RM14-RM243	10	5.31 (0.33)	−6.22 (0.13)	0.040	4.04	
		MQFeFG%1.2	RM128	80	11.67 (3.09)	−4.77 (0.29)	0.1001	10.01	
		MQFeFG%1.3	RM200- RM572	94.21	−1.57 (0.03)	−1.07 (0.01)	-0.003	-0.3	
		MQFeFG%1.4	RM572-RM220	130	−1.44 (0.01)	−3.05 (0.00)	0.0052	0.52	
	2	*MQFeFG%2.1*	RM6-RM341	135	−8.59 (10.71)	−14.08 (1.83)	0.1301	13.01	
	4	*MQFeFG%4.1*	RM255-RM335	55	−6.06 (10.04)	−11.46 (7.18)	0.0772	7.72	
	6	*MQFeFG%6.1*	RM528-RM586	6.01	5.98 (0.50)	4.82 (0.13)	0.0491	4.91	
		MQFeFG%6.2	RM528-RM586	24.01	3.91 (0.35)	2.22 (0.06)	0.0173	1.73	
Total#							0.41193	41.193	
GE	1	*MQFeGE1.1*	RM14-RM243	12	2.49 (14.85)	−7.63 (14.80)	0.023	2.3	
		MQFeGE1.2	RM572-RM220	152	15.01 (26.34)	40.07 (48.71)	0.69	69	
	2	*MQFeGE2.2*	RM208-RM526	0.01	−2.38 (10.70)	−12.42 (14.23)	0.022	2.2	
		MQFeGE2.3	RM6-RM341	133	−5.1356 (20.40)	15.16 (40.71)	0.107	10.7	
	3	*MQFeGE3.1*	RM7-RM473D	86.01	−4.8317 (38.82)	10.172 (38.70)	0.033	3.3	
	10	*MQFeGE10.1*	RM311-RM228	31.01	−12.5168 (7.94)	−7.6105 (40.63)	0.098	9.8	
	12	*MQFeGE12.1*	RM17-RM4A	4.01	−7.4507 (9.51)	−2.6601 (35.84)	0.018	1.8	
Total#							0.991	99.1	
Gve	1	*MQFeGVe1.1*	RM 243-RM246	50	0.0871 (1.07)	−1.1844 (179.89)	0.079	7.9	
		MQFeGVe1.2	RM128	80	−0.7334 (3.370)	−0.299 (3.25)	0.204	20.4	
		MQFeGVe1.3	RM572-RM220	159	19.7 (0.96)	−3.82 (1.53)	0.280	28	
	2	*MQFeGVe2.1*	RM6-RM341	119	1.293 (20.91)	0.9731 (20.94)	0.370	37	
	3	*MQFeGVe3.1*	RM520-RM426	54	0.258 (1.91)	−0.4112 (3.55)	0.031	3.1	
	4	*MQFeGVe4.1*	RM255-RM335	72	−0.623 (3.72)	−0.4329 (1.01)	0.029	2.9	
		MQFeGVe4.2	RM335-RM456B	101	0.1702 (8.400	−0.2872 (4.15)	0.014	1.4	

	6	*MQFeGVe6.1*	RM528-RM586	0.01	−0.2125 (1.27)	0.091 (1.69)	0.021	2.1	
		MQFeGVe6.2	RM314	24.01	−0.28 (2.19)	0.40 (0.20)	0.023	2.3	
	7	*MQFeGVe6.2*	RM560-RM70	22.01	−0.43 (2.38)	0.15 (0.35)	0.018	1.8	
		MQFeGVe1.3xMQGVe6.2			1.1972 (3.493)		0.060	6	AA
		MQFeGVe4.1xMQGVe6.2			−0.1832 (5.79)		0.003	0.3	AA
Total#							0.990	99	
SG	1	*MQFeSG1.1*	RM 243-RM246	57.01	−1.8113 (0.54)	5.5702 (1.52)	0.130	13	
		MQFeSG1.2	RM128	80.01	4.7962 (5.51)	−2.5449 (0.99)	0.150	15	
	4	*MQFeSG4.1*	RM551-RM255	50.01	1.7855 (0.75)	5.6734 (2.88)	0.130	13	
	6	*MQFeSG6.1*	RM539-RM454	15.01	2.7558 (1.59)	3.1008 (0.83)	0.080	8	
		MQFeSG1.2x MQFeSG6.1			6.016(1.77)		0.053	5.3	DA
Total#							0.560	56	
GPV	1	*MQFeGPV1.1*	RM 243-RM246	57.01	−0.8049 (0.54)	2.4748 (1.51)	0.130	13	
		MQFeGPV1.2	RM128	80.01	2.132 (5.51)	−1.1312 (0.99)	0.150	15	
	4	*MQFeGPV4.1*	RM551-RM255	50.01	0.7927 (0.75)	2.521 (2.88)	0.130	13	
	6	*MQFeGPV6.1*	RM539-RM454	15.01	1.2251 (1.59)	1.3799 (0.83)	0.080	8	
		MQFeGPV1.2x MQFeGPV6.1			2.67(1.37)		0.053	5.3	DA
Total#							0.560	56	
GC	1	*MQFeGC1.1*	RM 243-RM246	53.01	−6.87 (1.54)	31.03 (5.45)	0.390	39	
		MQFeGC1.2	RM128	80.01	12.30 (6.07)	−9.92 (1.93)	0.130	13	
	4	*MQFeGC4.1*	RM551-RM255	50.01	14.68 (1.97)	10.25 (0.65)	0.140	14	
		MQFeGC4.2	RM335-RM456B	62.01	−12.80 (1.24)	11.63 (0.24)	0.080	8	
		MQFeGC1.1x MQFeGC1.2			−12.87(1.66)		0.022	2.2	DA
		MQFeGC1.2 x MQFeGC4.2			7.77(1.48)		0.029	2.9	AA
Total#							0.800	80	
GI	1	*MQFeGI1.1*	RM243-RM246	53.01	0.989 (0.02)	54.38 (4.91)	0.180	18	
		MQFeGI1.2	RM128	80.01	2.37 (0.12)	−20.22 (2.68)	0.010	1	
		MQFeGI1.3	RM572-RM220	122.01	−56.88 (7.29)	58.77 (6.75)	0.700	70	
	2	*MQFeGI2.1*	RM526-RM327	35.21	−11.71 (4.20)	−4.91 (0.10)	0.007	0.7	
	3	*MQFeGI3.1*	RM520-RM426	50.01	−9.98 (1.04)	5.92 (0.20)	0.005	0.49	
	4	*MQFeGI4.1*	RM280-RM119	16.01	5.50 (0.34)	5.28 (0.09)	0.010	1	
		MQFeGI4.2	RM252-RM335	104.01	5.81 (0.19)	−15.33 (18.11)	0.010	1	
		MQFeGI1.1x MQFeGI2.1			8.30(0.40)		0.006	0.6	AA
Total#							0.940	94	
GV	1	*MQFeGV1.1*	RM 243-RM246	61.01	−5.09 0.39)	18.26 (1.79)	0.125	12.5	
		MQFeGV1.2	RM128	80.01	13.73 (3.57)	−9.02 (0.96)	0.134	13.4	
	6	*MQFeGV6.1*	RM528-RM586	0.01	6.08 (0.83)	11.21 (1.46)	0.077	7.7	
Total#							0.337	33.7	
MGT	1	*MQFeMGT1.1*	RM572-RM220	168.01	0.40 (0.20)	2.05 (2.50)	0.086	8.6	
	6	*MQFeMGT6.1*	RM528-RM586	2.01	1.64 (2.64)	−0.53 (2.01)	0.1	10	

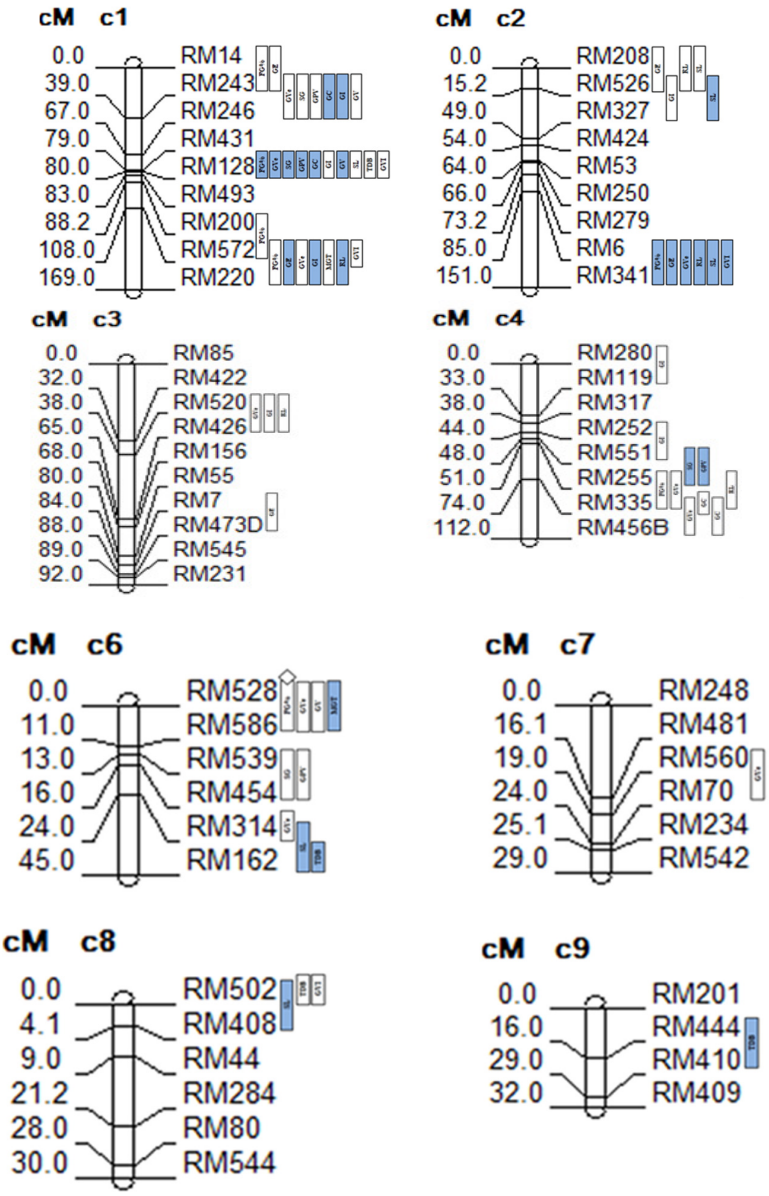
[a]Number of chromosomes, [b]QTL position from the first marker (cM),[c] Maximum likelihood LOD score for the individual QTL, [d]Allelic genetic effect, [e]Phenotypic variance explained by the individual QTL, [f]QTLx QTL, FG%= Final germination percentage, GVe = germination velocity, GE= Germination energy, SG=Speed of germination, GPV= Germination peak value, GI=Germination index, GC= Germination capacity, GV=Germination value, MGT=Mean germination time. The positive or negative additive or dominant values indicating the allele from 'Pokkali' or 'Pak basmati' increases the parameter respectively

Table 4. QTL detection by multiple interval mapping for growth parameters

Trait	[a]Chr No	[b]NO of QTLs	Marker interval	[c]Position	[d]Genetic Effect		R2	[e]R2 (%)	[f]VG
RL	*1*	*MQFeRL1.1*	RM572-RM220	145.01	0.53 (1.53)	−0.98 (5.77)	0.11	11	
	2	*MQFeRL2.1*	RM208-RM526	0.1	0.17 (3.08)	−0.67 (0.62)	0.01	0.1	
		MQFeRL2.2	RM208-RM526	6.01	−0.42 (2.28)	1.65 (0.39)	0.2	20	
		MQFeRL2.3	RM6-RM341	111.01	1.926 (10.39)	0.99 (1.23)	0.55	55	
	3	*MQFeRL3.1*	RM520-RM426	48.01	0.29 (1.57)	0.76 (5.17)	0.026	2.6	
	4	*MQFeRL4.1*	RM255-RM335	69.01	0.002 (0.01)	−1.103 (4.20)	0.09	9	
	11	*MQFeRL11.1*	RM20B-RM21	23.01	0.35 (1.85)	−0.11 (3.40)	0.009	0.9	
		MQFeRL1.1x MQFeRL11.1			1.16 (1.63)		0.036	3.6	*DD*
Total#							0.99	99	
SL	*1*	*MQFeSL1.1*	RM128	80.01	0.64 (4.54)	−0.31 (2.07)	0.051	5.1	
	2	*MQFeSL2.1*	RM208-RM526	2.01	0.95 (0.86)	−0.15 (0.45)	0.028	2.8	
		MQFeSL2.2	RM526-RM327	28.2	−1.57 (2.11)	2.01 (2.86)	0.243	24.3	
		MQFeSL2.3	RM6-RM341	111.01	1.54 (0.02)	1.50 (13.62)	0.274	27.4	
	6	*MQFeSL6.1*	RM314-RM162	36.01	1.63 (5.56)	−0.79 (1.11)	0.179	17.9	
	8	*MQFeSL8.1*	RM502-RM408	2.01	1.44 (4.75)	−0.50 (4.14)	0.119	11.9	
	11	*MQFeSL11.1*	RM20B-RM21	23.01	−1.16 (9.38)	−0.124 (1.70)	0.072	7.2	
	12	*MQFeSL12.1*	RM270-RM19	28.01	−0.33 (0.92)	−0.94 (1.86)	0.029	2.9	
Total#							0.99	99	
TDB	*1*	*MQFeTDB1.1*	RM128	80.01	0.075 (5.52)	−0.034 (0.69)	0.15	15	
	6	*MQFeTDB6.1*	RM162	44.01	0.032 (1.10)	0.049 (1.28)	0.062	6.2	
	8	*MQFeTDB8.1*	RM502	0.01	0.042 (2.14)	−0.010 (0.07)	0.055	5.5	
	9	*MQFeTDB9.1*	RM444-RM410	22.01	−0.055 (1.71)	0.081 (1.65)	0.146	14.6	
Total#							0.99	99	
GVI	*1*	*MQFeGVI1.1*	RM128	80.01	138.26 (2.48)	−16.96 (0.02)	0.081	8.1	
		MQFeGVI1.2	RM572	108.01	−135.60 (2.69)	−45.87 (0.19)	0.033	3.3	
	2	*MQFeGVI2.1*	RM6-RM341	101.01	67.91 (0.46)	537.47 (4.16)	0.53	53	
	8	*MQFeGVI8.1*	RM502	0.01	148.52 (3.56)	−102.31 (0.94)	0.098	9.8	
Total#							0.73	73	

[a] Number of chromosomes, [b]QTL position from the first marker (cM), [c] Maximum likelihood LOD score for the individual QTL, [d]Allelic genetic effect, [e]Phenotypic variance explained by the individual QTL, [f]QTLxQTL, RL=Root length, SL= Shoot length, TDB= Total dry biomass, GVI= Germination Vigour index. The positive or negative additive or dominant values indicating the allele from 'Pokkali' or 'Pak basmati' increases the parameter respectively

Multiple Interval Mapping (MIM)



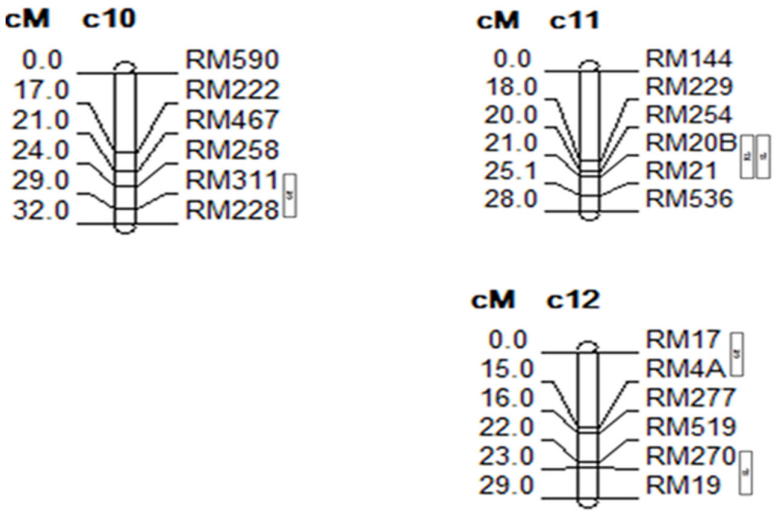


Figure 4. Fe^{2+} Chromosomal locations of QTLs by MIM analysis for FG%=Final germination percentage, GVe=germination velocity, GE=Germination energy, SG=Speed of germination, GPV=Germination peak value, GI=Germination index, GC=Germination capacity, GV=Germination value, MGT=Mean germination time, RL=Root length, SL= Shoot length, TDB=Total dry biomass, GVI=Germination Vigour index. The position of QTLs is indicated by vertical bars beside chromosomes. The vertical bars are drawn to be equal to the length detected for the QTL in the QTL Cartographer, and chromosome numbers are indicated above each chromosome. The symbol ◊ identifies two QTLs on same interval

Discussion

A significant effect of interaction between varieties and Fe^{2+} stresses was determined by ANOVA, mean and standard error of mean, indicating a differential response of varieties to Fe^{2+} stress. The severe effect of high Fe^{2+} toxicity to germination traits as compared to control indicating that high concentration of Fe^{2+} toxicities has a negative effect on rice growth and yield. Visible effects linked of high ferrous concentrations were found in 'Pak Basmati', 'Penderas', and 'Firat'. These effects were reduction in germination parameters and retardation of growth traits particularly lack of root elongation as shown in Figure 1. In this study, 'Pokkali' also found most tolerant and 'Pak basmati' was found most sensitive varieties to Fe^{2+} toxicity than other rice varieties in term of germination and growth parameters. Wu *et al.* (2014) found that under acute Fe^{2+} toxicity 'Pokkali' showed markedly higher tolerance than *IR29* in symptom score of leaf bronzing and growth development. Khan *et al.* (2013) also observed that 'Pak Basmati' was susceptible to abiotic stress such as heavy metals toxicity. Similar result have been observed by Javed *et al.* (2011) while working with identification of QTLs related to salinity tolerance at seedling stage with $F_{2:3}$ population lines derived from a cross between 'Pokkali', an Indica rice variety highly abiotic stress tolerance, and 'Shaheen basmati', an Indica rice variety sensitive to abiotic stresses (Javed *et al*., 2011).

Genetic studies for Fe^{2+} toxicity tolerance either by using classical or molecular approaches in rice have referred to complex inheritance and are governed by many genes (Dufey *et al*., 2015; Dufey *et al*., 2009; Shimizu, 2009; Wu *et al*., 2014). The goal of this research was to determine the QTLs linked with Fe^{2+} toxicity tolerance in segregating population.

Twenty-one major QTLs on chromosomes 1, 2, 3, 4, 6, 7, 10 and 12 affecting all germination traits may be used as indicators for Fe^{2+} toxicity tolerance at seed germination, as shown in Figure 4. Dufey *et al*. (2009) identified 28 putative QTLs associated with 11 vegetative parameters that were chosen as indicators of plant tolerance or sensitivity against Fe^{2+} toxicity (Dufey *et al*., 2009). Traits FG%, GE, and GVe showed a

considerably higher number of QTLs than SG, GPV, GC and MGT. Furthermore, the traits FG% and GVe had similar regions of chromosomes, although positions were slightly different, confirming a strong relationship between these traits.

Anuradha *et al*. (2012) identified 14 QTLs for Fe^{2+} and Zn toxicities using MIM located on chromosomes 1, 3, 5, 7 and 12. Fe^{2+} toxicity tolerance depends on several mechanisms, such as root and tissue tolerance mechanisms.

Dufey *et al*. (2009) identified 28 putative QTLs associated with 11 vegetative parameters chosen as indicators of plant tolerance or sensitivity against Fe^{2+} toxicity. The QTLs *MQFeGI1.3* and *MQFeGE1.2* on chromosome 1, are located in the same region in the RM572-RM220 interval, which affects the germination energy and germination index, showing large phenotypic variations of 69% and 70%, respectively, suggesting that these QTL regions may be important to Fe^{2+} toxicity tolerance at the germination stage, and this parameter could be chosen as an indicator of the degree of crop tolerance or sensitivity to Fe^{2+} toxicity. Anuradha *et al*. (2012) supported our findings while investigating QTL mapping and candidate genes for iron and zinc stresses in RILs derived from *Madhukar* × *Swarna* unpolished rice. They observed that high phenotypic variation and tolerance to Fe^{2+} toxicity did not follow a normal distribution.

Traits FG%, GE and Gve showed considerably higher numbers of QTLs than SG, GPV, GC and MGT. The strong relation of FG % and GVe was confirmed by MIM as FG%, and GVe had similar regions of chromosomes, although positions were slightly different. The GVe showed some new QTL regions but with low LOD and phenotypic variations compared to the set standard for major QTLs. Similarly, SG and GPV had identical QTLs, confirming their strong relationship. Two QTLs were detected for MGT as for the MIM analysis model. Tolerance to Fe^{2+} toxicity depends on several mechanisms, such as tissue tolerance, initial vigour, active oxygen species scavenging system and exclusion from roots (Becker and Asch, 2005). Rice roots diffuse oxygen in the surroundings of root media through arenchyma and convert Fe^{2+} into Fe^{3+} by oxidizing plaque on the root surface (Chen *et al*., 2006). Seven QTLs for RL identified in this study with phenotypic variation ranging from 0.1% to 55% on chromosomes 1, 2, 3, 4, and 11 indicated that these chromosomal regions may be important for root tolerance mechanisms to Fe^{2+} toxicity in rice. QTLs affecting rice shoots in terms of Fe^{2+} concentration were reported by Shimizu *et al*. (2009). Major QTLs for RL and SL with large phenotypic variation could be implicated in the determination of tolerance to ferrous iron toxicity. Three major QTLs, *MQFeSL2.2*, *MQFeSL2.3*, *MQFeSL6.1* and *MQFeSL8.1*, with 24.3%, 27.4%, 17.9% and 11.9% of the total phenotypic variations, respectively, were identified for SL on chromosomes 2, 6, and 8. Fe^{2+} ions induced activation of the enzymes glutathione reductase and ascorbate specific for the H_2O_2 scavenging system (Wissuwa *et al*., 1998). The major QTLs *MQFeTDB1.1*, *MQFeTDB9.1* and *MQFeGVI2.1* with trait variations of 15%, 14.6% and 53%, respectively, were detected for TDB and GVI. Wissuwa *et al*. (1998) detected QTLs for dry weight in backcross inbred lines (BILs) derived from *Nipponbare*/*Kasalath*//*Nipponbare* (*BC1F6*) and revealed that dry weight production under Fe^{2+} toxicity is viewed as an indirect indicator of tolerance to Fe^{2+} toxicity in rice. Several QTL regions were associated with more than one trait, suggesting a common genetic basis of traits called pleiotropic or closely linked genes (Dufey *et al*., 2009). The chromosomal region RM128 affected seven germination and growth traits that explained their high phenotypic variation of 13.4% to 20%, respectively. These findings suggest that trait correlation may be recognized to the effects of pleiotropy or to the very close linkage of genes (Huang et al., 2006). A similar result was reported by Dufey *et al*. (2009) while investigating novel QTLs in an interspecific backcross of *Oryza sativa* L. × *Oryza glaberrima* for resistance to iron toxicity in rice.

Two pairs of digenic epistatic interactions with R_2 values ranging from 0.3% to 6% were detected for trait GVe and one epistasis GE for SG and GPV. Two pairs of epistases for the GC trait indicated that these traits were largely controlled by the main effect of the QTLs. One pair of epistases (*MQFeRL1.1* × *MQFeRL11*) was identified for trait RL with a 3.6% R2 value. Wu *et al*. (2000) detected three pairs of epistasis loci in a different population, and the effects explained only approximately 20% of the total variation in root

length. Additive × additive epistatic interactions play a very important role in controlling toxic element concentrations in rice (Lui *et al.,* 2004). In this study, three main effects of QTLs were involved in epistatic interactions which confirm that the effects of single-locus QTLs are mostly dependent on alleles at other loci. Seven digenic interactions for Fe^{2+} and six for Zn were identified previously and explained 47.9% and 50.2% of the total variation, respectively (Lui *et al*., 2004). The large effect of the interactions suggests that the regulation of element concentration is significant but complex, involving multiple genes in the uptake, transport and seed loading of elements. However, single genes have been shown to have major effects on Fe and Zn concentrations, and such genes may well underlie the major effects in QTLs (Johnson *et al*., 2011).

Conclusions

In our study, we used 400 SSR markers for identification of polymorphic markers 84 highly polymorphic markers were used for the detection of QTLs associated with tolerance to Fe^{2+} toxicity to specific chromosomal locations on a genetic linkage map. Seventy-two QTLs with phenotypic variations of 0.7% - 72.7% for all traits of germination and seedling growth were identified. A similar number of QTL × QTL interactions were explored in this study, which could provide us with an understanding of the factors essential for tolerance to Fe^{2+} toxicity in rice. The main QTLs for germination and growth parameters linked with Fe^{2+} toxicity tolerance would provide the opportunity to introduce marker-assisted recurrent selection (MAS) to the breeding programs.

Authors' Contributions

NJ and MJ designed the study. N.J developed populations. NJ and MAJ performed data analyses. AK and NJ wrote and revised the manuscript. SP and IA took part in some library work. All authors read and approved the final manuscript.

Ethical approval (for researches involving animals or humans)

Not applicable.

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Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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