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PHENOTYPIC AND GENOTYPIC SCREENING OF F_2 AND F_3 GENERATIONSIN RICE (*ORYZA SATIVA* L.) FOR SUBMERGENCE TOLERANCE

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ABSTRACT

Pedigree-based selection method combined with marker-assisted selection (MAS) provides a suitable resource for deriving elite lines with more favorable characters. The aim of the present investigation was to analyze the using field experiments and molecular markers we evaluated diversity of rice advanced $F_2$and F_3lines derived from cross ADT46/ Swarna sub1 aimed to select elite line(s) with favorable characters[High yielding variety / resistance to the submergence tolerance (sub1 genes)]. Selection for several important traits, including resistance to high yielding, resistant to submergence tolerance (SUB1 gene) was conducted during 2 and 3 generations. Genetic diversity of F_2 and F_3lines was studied using 288 and 11 polymorphic loci produced by long AP-PCR primers. Results showed that there was a great diversity within and between studied advanced lines. Average gene diversity across polymorphic loci for the two generations was 20.3% and 8.5%, respectively. Phenotypic evaluations in combination with MAS helped us to identify among the F_2segregants phenotyped for submergence tolerance, 84 per cent of the individuals were survived after de-submergence as compared to 90 per cent of survivability in resistant donor viz., Swarna Sub1 and FR13A. Our results markedly show that selection made by breeder has diverse effects on genetic structure of plant material, particularly in favor of fixating genetic background of superior parent.

KEYWORDS: *Phenotypic and Genotypic Screening, Polymorphic Loci, SIB Analysis, Submergence Tolerance*

Article History

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INTRODUCTION

Rice *(Oruza sativa L.)* is the major staple food for more than half of the world population and 90% of it is being produced and consumed in Asia. It is the major crop in the most flood- prone areas of South and South-East Asia. Flash flood or submergence is a common phenomenon in mainly lowland areas, subject to monsoon rains, seriously affecting crop establishment as well as survival, leading to severe yield losses. It imposes a complex abiotic stress in flood-prone ecosystem, because it substantially reduces crop stand, especially if it occurs during early vegetative stage and prolongs for more than a week. In India, the annual production of 85.5 million tons of rice with an average productivity of 1.9 t/ha comes from an area of 44.5 million ha spread over several ecologies of which about 17 million/ha is rainfed, drought and submergence prone. Flooding induced submergence results in substantial yield loss in rice which depends upon plant type, crop growth stage and the intensity and duration of submergence. Plant

survival during submergence also depends upon light intensity and flood water characteristics namely turbidity, temperature, turbulence and pH (Srivastava *et al.*, 2007).

As with other major abiotic stresses, breeding and selecting successful submergence tolerant rice cultivars have not yet met with notable commercial success till some years ago. Germplasm survey revealed the existence of limited amount of genetic variation for submergence tolerance. Intensive efforts at IRRI, Philippines resulted in the identification of a flood tolerant rice line called "FR13A" which showed tolerance up to 14 days of flooding. Exploitation of this genetic material in various breeding programs and mapping studies led to the understanding of genetic and molecular basis of improved submergence tolerance in this rice genotype. Submergence tolerance in FR13A is controlled by a putative Ethylene responsive Factor (Xu *et al*., 2006) located in the region Sub1 on chromosome 9 (Xu and Mackill, 1996). At IRRI, introgression of Sub-1 locus into a high yielding submergence susceptible Indian variety "Swarna" was successfully carried out through marker assisted whole genome selection. The improved Swarna called "Swarna Sub-1" showed improved level of tolerance to submergence than the original Swarna and it possessed all the other desirable attributes of Swarna (Neeraja *et al.*,2007). This report clearly showed the possibility of improving submergence tolerance in rice through marker assisted introgression of Sub1 locus. The strategy of marker assisted introgression of target locus through marker assisted pedigree selection improves the efficiency of selection. Marker-assisted pedigree selection would be especially effective for the transfer of recessive genes since their classical transfer requires additional recurrent selfing generations, a procedure that is prohibitively slow for most commercial breeders (Welz and Geiger, 2000). To date, the most economic and sustained way to overcome the problem of submergence stress is to develop submergence tolerant varieties. In the present investigation, therefore the possibility of developing submergence tolerant and high yielding varieties through marker assisted pedigree selection involving submergence tolerant genotypes was explored through selection in the segregating generations like F_2 and F_3.Based on these facts, the present study was undertaken with the following objectives:

To asses most discriminating molecular markers for submergence tolerance QTL sinF_2 and F_3 generations.

MATERIAL AND METHODS

Phenotypic Screening of F_2 Generation for Submergence Tolerance

The study was conducted at the Pandit Jawaharlal Nehru College of Agriculture and Research Institute, Karaikal. The F_2 population comprising of 1420 plants of the cross ADT46 / Swarna sub1 were raised along with their parents *viz.*, ADT46, Swarna sub1 and IR42 (susceptible check) and have been phenotyped for early crop stage submergence in submergence pond during August–January 2013-2014, Submergence was done on 25th days after transplanting for a period of 14 days(**table 1**). During the submergence period pH, EC and temperature were monitored on daily basis and the pond was de-submerged when the susceptible check IR42 started showing the symptoms of browning and withering of shoots. Then the de-submergence was done. De-submerged plants were then given 14 days to recover. Survival of the plants following de-submergence was scored in terms of elongation of existing leaves, development of new leaves, color of leaves at 14days after de-submergence. Scoring was done based on the modified Standard Evaluation System (SES) developed by Suprihatno and Coffman (1981) for rating submergence tolerance in rice. (**table 2**) The methodology adopted was given in detail in flow chart (**figure 2**). True F_1 s confirmed with SSR markers (**figure 1**)

Genotypic Screening of F_2 Generation

Leaf samples were collected from the plants which have scored for 1 and 3 and DNA was extracted from those young leaves collected from the tolerant progenies and along with susceptible variety.

Table 1: Details of Parents

Sl. No	Characters	ADT46	Swarna Sub1
1	Parentage	ADT38 / CO45	Swarna/FR13A
2	Type of germplasm	Cultivar	Cultivar
3	Season	Thaladi	Thaladi
4	Duration(days)	130-135	135
5	Rice grade	Medium slender	Short bold
6	Rice colour	White	White
7	Tolerance to submergence stress	Susceptible	Tolerant

Table 2: Score for Submergence Tolerance (Modified after Suprihatno and Coffman (1981)

Leaf Morphology	Score
Erect dark green leaves, greater elongation, new leaf development	1
Erect green leaves, little elongated	2
Green leaves, little elongation	3
Droopy, pale green leaves, moderate elongation	4-6
Long, pale green leaves, elongated, few survived	7-8
Long brownish leaves, elongated, dead	9

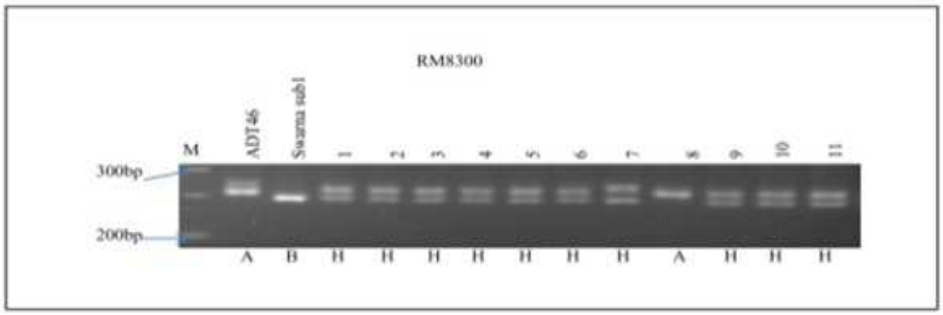

Figure 1: Marker Analysis of True F_1's.

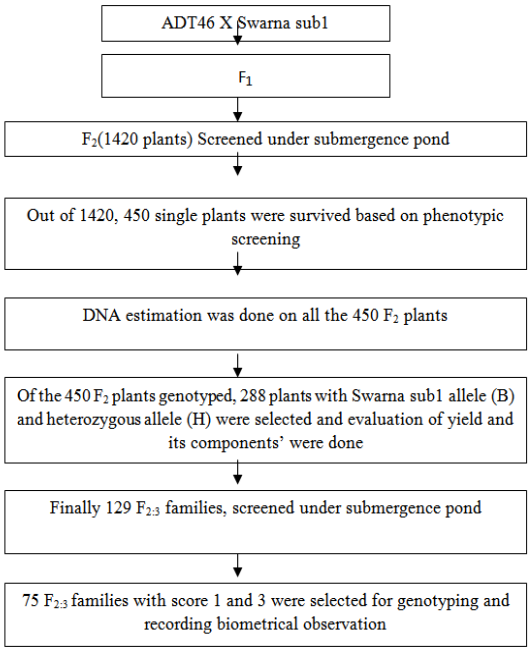

Figure 2: Flow Chart for Pedigree Selection.

Selection of $F_{2:3}$ Generation

A total of 450 F_2 plants which have scored 1 and 3 considered for selection and forwarding to F_3 generation. Of the 450 F_2 plants. 288 plants, which were scored for 1 and 3 with B and H alleles were selected. Out of 288 F_2 plants, 129 plants were phenotypic ally selected based on score 1 and 3 coupled with significant grain yield per plant over the general mean. These 129 plants were selected forwarding to F_3 generation, since these plants also showed the presence of Swarna sub1 specific allele in homozygous (BB) and heterozygous (AB) conditions based on genotyping.

Amplification of Genomic DNA using SSR Primers through Polymerase Chain Reaction (PCR)

The genomic DNA of the different rice genotypes isolated as described earlier were subjected to PCR amplification in thermal cycler (Eppendorf. AG. Germany) the reaction volume of 15 µl containing 2 µl of genomic DNA, 1X assay buffer, 200 mM of deoxyribonucleotides, 2 µM of $Mgcl_2$, 0.2 µM of each primer (both forward and reverse primers), 1 unit of Tag DNA polymerase (Bangalore Genei Pvt. Ltd., Bangalore) and 4.6 µl of sterile water. The PCR profile adopted was: (i) initial denaturation at 95^{o}C for 2 minutes, followed by (ii) 34 cycles of denaturation at 94^{o}C for 1 min, annealing at 55^{o}C and extension at 72^{o}C for 2 minutes and (iii) final extension at 72^{o}C for 10 minutes and at 4^{o}C for cooling. Annealing temperature was standardized for each primer and adopted for all the primers used in the study as identified by their specific Tm requirement.

Electrophoresis

The amplified products were separated in 3 percent agarose gel prepared in 1 XTBE buffer stained with Ethidium Bromide (0.5µg / ml). The gel was run in 1 XTBE buffer (0.89 M Trisborate, 0.02 MEDTA, pH8.0) at constant voltage of 85V for a period of 23 hours. The gel was visualized in UV transilluminator and photographs were taken using gel documentation system (Model Alpha Imager 1200, Alpha Innotech Corp., and USA).

Scoring

For each marker, allelic bands were scored based on tolerant and susceptible parental bands of the amplified products and were designated as A, B and H for homozygous susceptible, homozygous tolerant and heterozygote respectively. Data was entered in to an excel spread sheet and analyzed.

RESULTS AND DISCUSSIONS

A raising global population requires increasing crop production and research conducted so far suggests that the rate of increase in crop yields is currently declining and traits related to yield, stability and sustainability is considered as a major focus in plant breeding efforts. These traits include durable biotic, abiotic stress tolerant and other agronomic factors like water use efficiency and nutrient management. Pedigree based selection method combined with marker assisted selection provides a suitable resource for deriving elite lines with favorable characters. Molecular marker assisted selection involves selection of plants carrying genomic region that are involved in the expression of traits of interest through molecular markers. DNA based markers can be effectively utilized for tracing favorable allele(s) (dominant or recessive) across generations and identifying most suitable individual(s) among the segregating progeny based on allelic composition across a part or the entire genome. Pedigree marker assisted selection (MAS) is relevant for crops such as rice, where pedigrees of elite germplasm are known.

Table 3: Details of Polymorphic SSR Markers used for PCR Amplification of F_2 and F_3 Populations

Sl. No.	Marker Name	Primer Sequence	Product Size	Position (Mb)	References
1	RM8300	GCT AGT GCA GGG TTG ACA CA CTC TGG CCG TTT CAT GGT AT	209	6.6 Mb	Matsumoto *et al.* (2005)
2	ART5	CAG GGA AAG AGA TGG TGG A TTG GCC CTA GGT TGT TTC AG	217	6.39 Mb	Xu *et al.* (2006)
3	Sub1BC2	AAA ACA ATG GTT CCA TAC GAG AC GCC TAT CAA TGC GTG CTC TT	400	6.38 Mb	Xu *et al.* (2006)

Identification based on morphological character is time consuming and requires extensive field trials and evaluation. In addition, morphological differences may be epigenetic-or genetic-based characters. Molecular markers due to their advantages against to morphological and biochemical markers such as their plentifully, independence of tissue or environmental effects, diversity identification and selection in the earlier stages of plant development, can be a useful complement to morphological and physiological characterization of plants.

Despite empherical cross validation studies showing the advantage of molecular markers, there is still a need to test these results in actual breeding programme and inferences from those comparisons are important because the response to selection is dependent on the level of genetic variance for any trait (Falconer and Mackey, 1996)

Keeping these above in view, phenotypic selections coupled with marker assisted selection were attempted for submergence tolerance in F_2 and F_3 segregating generations (ADT46 / Swarna Sub1 cross). The objective of the study was to compare marker based phenotypic strategy for the confirmation of Sub1 QTL, identified in chromosome 9 of rice. The phenotypic based selection in segregating progeny were confirmed for the presence of Sub1 locus through marker assisted selection using a gene specific DNA marker Sub1BC2 along with flanking marker RM8300 and ART5 in F_2 segregating populations for selection of individuals to be forwarded to F_3 generation.(**table 3**)

The present study was undertaken with the objectives of identifying submergence tolerance individuals from the cross between a popular rice variety ADT46 and submergence tolerant variety, Swarna Sub1. The parental polymorphism was tested between ADT46, Swarna Sub1, FR13A and susceptible variety, IR42. The PCR amplified products of three markers were analyzed for the presence or absence of the Sub1 allele in the above genotypes. The RM8300 amplicon from Swarna Sub1 and FR13A were 230 bp as compared to 200 bp in ADT46 and IR42 as compared to 200 bp and 230 bp from Swarna Sub1, FR13A and ADT46, IR42 in the ART5 amplicon. Higher allelic variability for ART5 marker as compared to RM8300 was observed among the varieties tested. However, Sub1BC2 amplicon from Swarna Sub1 and FR13A were 230 bp as compared to 270 bp in ADT46 and IR42.

The FR13A and Swarna Sub1 were found to be superior to ADT46 and IR42 in terms of submergence tolerance. Accurate phenotyping of submergence tolerant, intolerant and susceptible varieties were assessed by acclimative shoot response during 14 days of submergence period by determining the survivability 14 days after de-submergence period.

During the 14 days of submergence, ADT46 and IR42 showed a significant increase in plant height compared to that of submergence tolerant varieties *viz.*, Swarna Sub1 and FR13A. The F_2 and F_3 individuals selected based on phenotypic and genotypic data showed similar acclimative shoot response as in submergence tolerant varieties (**figure. 3**). Leaves of the susceptible varieties appeared spindly, chlorotic and elongated whereas leaves of submergence tolerant varieties showed very little elongation and were fully green in colour.

Evaluation of 1420 F_2 plants under submergence pond revealed the presence of continuous variation for the targeted quantitative traits *viz.*, plant height before submergence, plant height after submergence, days to flowering, plant height at maturity, number of productive tillers per plant, panicle length, panicle weight, 100 grain weight and grain yield per plant. This continuous variation existed among the population is indicative of suitability of the populations for effecting efficient selection from the early stages of segregating generations.

In the present study, integration of genotypic and phenotypic data for selection of individual plant in F_2 generation has resulted in the identification of 288 single plants for forwarding to next generation. To have efficiency in identifying best individuals to be forwarded for further study, attempt has been made with data on quantitative trait with genotyping of individual plant. Of the 288 individuals, 129single plants were selected which conforms the tolerance to submergence (plants with Swarna Sub1 alleles (B) and Heterozygous alleles (H)) coupled with increased significant grain yield per plant.

SIB ANALYSIS

Development of submergence tolerant rice from any rice variety / germplasm via the use of DNA marker to assist selection in rice breeding allows the breeders to circumvent several inherent problems associated with conventional breeding especially those involving cumbersome process and time consuming submergence tolerance phenotypic screening.

In this present investigation, recovery level after de-submergence of F_3 families / individuals were phenotyped as well as genotyped. A total of 129 sibs were evaluated for submergence tolerance besides for quantitative traits. The investigation on quantitative traits showed the presence of continuous variation for all the traits studied.

Sib analysis of all the 129 sibs under field condition revealed that the recovery level after de-submergence was varying, where all the plants carrying lines with ADT46 alleles were dead in the sibs identified as containing H allele in F_2 generation.

In order to identify the Swarna Sub1 allele, it was planned to employ the Sub1BC2 for background selection which will lead to accelerated recovery of submergence tolerance, assuming the phenotypic association of submergence tolerance with Sub1 locus.

There are several instances when phenotypic screening can be strategically combined with marker assisted selection. Several studies indicate that the combined marker assisted selection is more efficient than phenotypic screening alone, especially when large population sizes are used and trait heritability is low (Hospital *et al*., 1997). In a typical plant breeding programme, normally early generation marker assisted selection is great advantageous in nature because plants with undesirable gene combinations can be eliminated which allows the breeder to focus attention on lesser number of objective based on lines in future generations. Several studies postulated that the single marker assisted selection step could be performed on F_2 and F_3 populations derived from elite parental combinations.

In a self pollinated crop like rice, the important aim is to fix allele in their homozygous state as early as possible when using co-dominant DNA marker it becomes possible to fix the specific allele in the homozygous state as early as in F_2 generation.

In the present investigation, attempts were made to combine Sub1BC2 marker (tight linked to submergence tolerance) data, submergence tolerance score and yield components in F_3 families by way of sib analysis. Among the 129 sibs evaluated for submergence tolerance, 75 sibs were survived after de-submergence. The remaining sibs even though

phenotyped and genotyped as carrying either B or H alleles have completely dead due to the reason that the Sub1 locus allele would have been expressed to a lesser level that could not withstand submergence.

A selection pressure was applied to reduce the sibs to be forwarded to $F_{3:4}$ generations. To increase the efficiency of selection, a sib analysis score chart was prepared by giving score 1 wherever the quantitative traits studied attained a positive with significant level besides the favorable allelic score of sibs (**table 4**). The allelic score revealed the presence of either complete B allele or H and B allele individual plants among the 75 sibs. Of the 75 sibs, eleven sibs have recorded highest score of 5 and above which includes significant level for grain yield per plant. These sibs are worth considering for forwarding to next generation for further evaluation. Preliminary results of this study indicated the potential use of combined marker assisted pedigree selection over either of the method in isolation to improve the submergence tolerance in the early generation breeding materials derived from elite parental combinations.

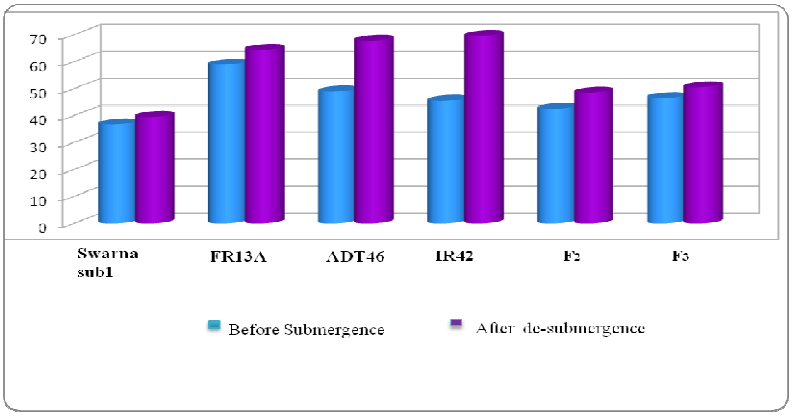

Figure 3: Plant Height (cm) of Parents, F_2 and F_3 Generations.

Table 4: Sib Analysis Score Chart

Sl.No	SibNo	Characters									Total	Allelic score
		Plant Height before Submergence (cm)	Plant Height after Submergence (cm)	Days to Flowering	Plant Height at Maturity (cm)	Number of Productive Tillers/Plant	Panicle Length (cm)	Panicle Weight (g)	100 Grain Weight (g)	Grain Yield /Plant (g)		
1	11	-	-	-	-	1	-	1	-	1	3	B
2	56	-	-	-	-	1	-	1	-	-	2	B
3	85	-	-	1	1	1	1	1	-	1	6	H,B
4	86	-	-	1	-	1	1	-	-	1	4	B
5	131	-	1	-	1	1	1	-	-	-	4	B
6	151	-	-	-	1	1	1	1	-	-	4	B
7	160	-	1	-	-	1	1	1	-	-	4	B
8	166	-	-	1	1	1	-	-	-	-	3	H,B
9	180	-	-	-	-	-	1	1	-	-	2	B
10	182	-	-	-	-	1	-	-	1	-	2	H,B
11	181	-	-	-	1	1	-	-	-	-	2	B
12	201	-	-	1	-	-	-	1	-	-	2	B
13	221	-	-	-	-	-	-	1	1	1	3	B
14	224	1	1	-	1	-	1	-	-	1	5	B
15	230	-	-	-	-	1	1	1	-	1	4	B
16	234	-	-	1	-	-	1	1	1	1	5	B
17	239	-	-	1	-	-	-	-	1	1	3	H,B
18	241	-	-	-	-	1	1	1	-	-	3	B
19	247	-	-	1	-	1	-	-	-	-	2	H,B
20	254	1	1	1	-	-	1	-	1	-	5	H,B
21	258	-	-	1	1	-	1	-	-	-	3	B
22	273	1	-	1	-	1	1	-	-	1	5	B
23	281	-	-	1	1	1	-	1	-	-	4	H,B
24	284	-	-	1	1	-	1	1	1	1	5	B
25	293	1	-	1	1	-	-	1	-	-	4	H,B
26	307	1	-	1	1	-	1	-	1	-	5	B
27	310	1	-	1	-	-	1	1	1	-	5	H,B
28	331	-	-	1	1	-	1	-	1	-	4	B
29	339	1	-	-	1	-	1	-	-	-	3	B
30	346	1	-	-	1	-	1	1	-	-	4	B
31	351	1	-	1	-	1	1	-	1	-	5	B
32	354	1	1	1	1	-	-	1	1	1	7	B
33	356	1	-	1	1	-	-	-	-	1	4	H,B
34	370	1	-	1	1	-	-	-	-	1	4	H,B

Table 4: Contd.,

35	379	1	1	1	1	-	1	-	-	1	6	B
36	380	-	-	1	1	1	1	1	1	-	6	H,B
37	381	-	-	1	-	-	-	1	1	-	3	B
38	390	1	1	-	-	-	1	-	-	-	3	H,B
39	396	1	1	1	1	-	1	1	1	-	7	B
40	399	1	-	1	1	-	-	-	1	-	4	B
41	419	-	-	-	-	-	1	-	-	1	2	H,B
42	431	1	-	1	1	1	-	-	1	1	6	B
43	435	1	1	1	1	-	1	1	1	-	7	B
44	443	1	1	-	1	-	-	-	1	1	5	B
45	495	-	1	-	-	-	-	-	-	-	1	B
46	521	1	1	-	-	1	-	-	-	-	3	B
47	531	1	1	-	1	-	-	-	1	-	4	H,B
48	599	-	1	-	-	-	-	1	-	1	3	B
49	610	1	1	-	1	-	-	-	-	-	3	H,B
50	650	1	1	-	-	-	1	-	1	-	4	B
51	680	1	1	-	1	-	1	-	1	-	5	B
52	690	1	-	-	-	-	-	-	-	-	1	B
53	692	1	1	-	-	-	1	-	-	1	4	H,B
54	859	1	1	-	-	-	-	-	1	1	4	H,B
55	860	1	-	-	1	-	1	-	-	1	4	B
56	861	-	1	-	-	-	-	-	-	-	1	B
57	922	-	1	1	1	-	-	-	-	1	4	B
58	980	1	1	-	-	-	-	1	-	-	3	B
59	1017	1	1	-	-	-	-	1	-	-	3	B
60	1040	-	1	1	-	-	1	-	-	-	3	B
61	1044	1	1	-	1	1	-	1	-	-	5	B
62	1110	-	1	1	-	1	-	1	-	1	5	B
63	1180	-	1	1	-	1	-	-	-	-	3	H,B
64	1191	-	1	-	-	1	1	-	-	-	3	B
65	1199	1	1	-	-	-	-	-	-	-	2	B
66	1210	-	-	-	-	1	-	1	-	-	2	B
67	1213	-	-	1	-	1	-	-	-	-	2	B
68	1221	-	1	-	-	1	1	1		1	5	B
69	1231	1	1	-	-	-	1	-	1	-	4	B
70	1272	-	-	1		1	1	-	1	-	4	B
71	1285	1	-	-	1	1	-	1	-	-	4	B
72	1288	-	1	1	-	-	1	1	1	1	6	B
73	1295	-	-	-	-	1	1	1	-	1	4	H,B
74	1311	-	1	1	-	-	-	1	-	1	4	B
75	1314	-	1	-	-	-	-	1	1	-	3	B

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APPENDICES

Figure 4: Phenotyping of F_2 Plants in Submergence Pond before Submergence.

Figure 5: F_2 Generation under Submergence for 14 Days.

Figure 6: De-Submerged Field View after 14 Days of Submergence.

Figure 7: Symptoms of Submergence Injury in F_2 Generation (14^{th} day after De-Submergence).

Figure 8: Phenotyping of F_3 Families in Submergence Pond before Submergence.

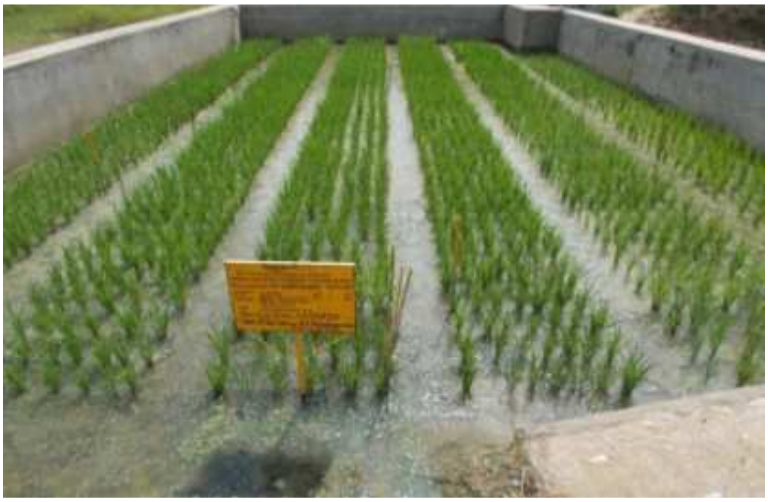

Figure 9: F_3 Generation under Submergence for 14 days.

Figure 10: De-Submerged Field view after 14 days of Submergence.

Figure 11: Symptoms of Submergence Injury in F_3 Generation (9^{th} day after de-Submergence).

Figure 12: Symptoms of Submergence Injury in F_3 Generation (35^{th} day after Desubmergence).