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Molecular and phenotypic screening for submergence tolerance in lowland rice

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ABSTRACT

Genetic variability was studied for yield and yield related traits in fifty one elite low land rice cultures, selected on the basis of their tolerance to submergence and stagnant flooding. The experimental materials possessed considerable amount of variability for all the traits. The genotypes were grouped into three clusters with ten sub-clusters on the basis of presence or absence of specific bands by using Sub1 specific SSRs markers. The cultivars like Savitri-Sub1, IR 64-Sub1, PSBRc 18-Sub1, Swarna-Sub 1, IR 85086-SUB 33-3-2-1, IR 87439-BTN-145-2-1, IR 88228-33-3-5-2, IR 88234-STG 11-1-1-1, IR 88250-20-1-1-3, IR 88764-SUB 30-1-1-2 and IR 88776-SUB 8-1-1-2 had shown high level of submergence tolerance through phenotyping. Submergence tolerance in these these lines were further validated through molecular screening using SSR markers. Amongst the molecular markers used, Sub1A 203, a direct marker is better for differentiating tolerant from intolerant to submergence as compared to AEX, Sub1BC2 and RM 8300 for marker-assisted breeding program. Besides, it may be inferred that role of Sub1B and Sub1C may be ignored for submergence tolerance.

Key words: *Phenotyping, submergence tolerance, genotyping, SSRs markers, molecular divergence*

INTRODUCTION

Rice being the staple food for more than 70 per cent Indians and a source of livelihood for 120-150 millions rural households, the requirement of rice production by 2030 would be around 145 million tonnes from the present level of 105 million tonnes to sustain self-sufficiency in rice. More than 60% of rice produced in India comes from Eastern India. Out of the 26.8 mha rice area in eastern India, rainfed lowland rice constitutes 39% of the total rice area. About 8.0 mha of rainfed lowland areas are flood/submergence prone (Reddy et al., 2013). Rainfed lowlands constitute highly fragile ecosystems, always prone to flash-floods and stagnant flooding submergence stress situations. Since submergence and stagnant flooding stresses are unpredictable, therefore, there is a need to develop new varieties with high yield and tolerance to both submergence and stagnant flooding for greater stability of production under the diverse rainfed lowland ecosystems of eastern Indian states. The present investigation was, therefore aimed at evaluating fifty one such elite lowland rice cultures. Different yield attributing traits were examined to study the availability and extent of genetic variability in the experimental material. The test genotypes were screened for tolerance to submergence flooding through appropriate screening test under control condition and validation was done by SSRs markers to confirm the presence of *Sub1A* gene for submergence tolerance. The genotypes were also grouped into various clusters according to presence or absence of *Sub1A* gene by using Sub1 A specific SSRs markers.

MATERIALS AND METHODS

The experimental material used in the present

investigation consisted of fifty one elite low landrice genotypes including Sub1 introgressed lines along with their parents, elite lines combining submergence tolerance with stagnant flooding, promising donors and four check varieties. The test genotypes were evaluated in a Randomized Block Design with two replications with a spacing of 20 x 15 cm at Rice Research Station, OUAT, Bhubaneswar during *Kharif*, 2014 under recommended cultural practices. Observations were recorded on 5 randomly selected plants for different yield attributing traits. The data were analyzed by using ANOVA (Panse and Sukhatme, 1954). The 51 genotypes comprised of commonly cultivated lowland cultivars and fixed lines obtained from the International Rice Research Institute, Manila, Philippines were screened for submergence tolerance. Seeds of the 51 genotypes were direct seeded in pots that were submerged in the tanks at 20 day- seedling stage with two replications. Water depth of 1m was maintained in the screening tanks during submergence period. When the susceptible check showed maximum leaf damage i.e., after about 14 days of complete submergence, tanks were de-submerged and the survival of plants was scored after 14 days of recovery (Fig. 2). The genotypes were scored as per standard evaluation system (SES) for rice developed by International Rice Research Institute, Manila, Philippines. Gene-based and intragenic Sub1 DNA markers based on DNA sequences published by Xu et al., 2006 and available in the NCBI database (www.ncbi.nlm.nih.gov) were used for genotyping of the lowland cultivars. The genotyping works were carried out at Molecular Breeding Lab.-1, ICAR-National Rice Research Institute, Cuttack, Odisha. The genomic DNA was isolated from 10 days old seedling following CTAB method as per Murray and Thompson 1980. DNA amplification was performed in a Gradient Thermal Cycler (Verity, Applied Bio Systems) with a reaction volume of 20 l containing 1.5 mM Tris HCL (pH 8.75), 50 mM KCL, 2mM $MgCl_2$, 0.1% TrotonX-100, 200µM each of dATP, dCTP, dTTP, dGTP, 4pMole of each forward and reverse primers, 1 unit of Taq Polymerase and 30ng of genomic DNA. The reaction mixture was initially denatured for 4 mins at 94 ^{0}C and then subjected to 35 cycles of 1 min denaturation at 94 ^{0}C, 1 min annealing at 55 ^{0}C, and 1 min extension at 72 ^{0}C; and then a final extension for 10 mins at 72 ^{0}C. Aliquots of 10l of DNA products from PCR amplification were loaded in 3 % agarose gel containing 0.8 g/ml Ethidium Bromide for electrophoresis in 1X TBE (pH 8.0). At least one lane was loaded with 50bp DNA ladder. The gel was run at 60 volts (2.5V/cm) for 4 hrs and photographed using a Gel Documentation System (Syn Gene). Data were scored for analysis on the basis of the presence or absence of the amplified products for each genotype-primer combination. The data entry was done into a binary data matrix as discrete variables. Data were analyzed and similarity matrix was constructed from binary data with Jaccard's coefficients and dendrogram was generated with unweighted pair group method arithmatic average (UPGMA) algorithm, using FreeTree software (Hampl et al., 2001; Pavalice et al., 1999) and the dendrograms were visualized by Tree view 32 software (Page,1996). Principle component analysis (PCA) analysis (Pradhan et al., 2016; Pandit et al., 2017) was used to estimate Euclidean distance between genotypes and correlation between the variables. These analyses were performed using SAS programs (Burgeano et al., 2000). The association study between the Sub1 markers and phenotyping parameters was done with Tassel 5 (Bradbury et al., 2007).

RESULTS AND DISCUSSION

The analysis of variance showed significant variation existed among the test genotypes for all the traits studied, indicating presence of substantial genetic variation and thus provides enough scope for effective selection (Table 1). The phenotyping results (Table 2) for plant survival indicated that 11 out of 51 genotypes

Table 1. Analysis of variance for quantitative characters in 51 lowland rice breeding lines.

Sl. No.	Characters	Mean sum of squares		
		Replic-ation(1)	Genot-ype(50)	Error (50)
1.	Days to 50% flowering	102.01	264.81**	78.35
2.	Plant height (cm)	15.55	163.37**	10.23
3.	Panicle length (cm)	2.12	6.86**	0.80
4.	Panicle number	5.29	4.17**	2.57
5.	No. of fertile grains/panicle	642.68	995.40**	334.85
6.	Fertility percentage	3.67	82.47**	22.45
7.	100-grain weight (g)	0.003	0.34**	0.044
8.	Harvest index	0.001	0.009**	0.003
9	Grain yield / plant (g)	11.77	22.54**	9.90
10.	Plot yield (q/ha)	70.42	112.58**	18.15

* and ** Significant at 5% and 1% level of probability respectively. Figures in parentheses indicates degrees of freedom (df) for corresponding sources of variation.

Table 2. Phenotyping of 51 rice genotypes for submergence tolerance.

Sl. No.	Name of the genotype	Total tillers before submergence	Regenerated tillers after 14 days of de-submergence	% survival	SES score	Remarks
1	BR 11	5	0	0	9	S
2	BR 11-Sub1	6	4	67	7	MS
3	Ciherang	24	0	0	9	S
4	Ciherang-Sub1	19	15	78.9	5	M T
5	Savitri	23	0	0	9	S
6	Savitri-Sub1	12	12	100	1	T
7	IR 64	22	0	0	9	S
8	IR 64-Sub1	22	22	100	1	T
9	PSRBc 18	18	1	5.5	9	S
10	PSBRc 18-Sub1	11	11	100	1	T
11	Samba Mahsuri	16	0	0	9	S
12	Samba Mahsuri-Sub1	8	5	62.5	7	MS
13	Swarna	26	2	7.7	9	S
14	Swarna-Sub1	16	16	100	1	T
15	Thadokkham	9	1	11.1	9	S
16	Thadokkham- Sub1	16	11	68.8	5	M T
17	Inpara-3	19	17	89.5	5	M T
18	IR 42	24	0	0	9	S
19	IR 68	13	2	15.4	9	S
20	IR 72	5	0	0	9	S
21	IR 74	27	6	22.2	9	S
22	PSBRc 68	18	15	83.3	5	M T
23	PSBRc 70	18	4	22.2	9	S
24	PSBRc 102	21	4	19.05	9	S
25	NSICRc 214	28	0	0	9	S
26	OR 142-99	18	2	11.1	9	S
27	Pratikshya	20	0	0	9	S
28	Mahanadi	14	8	57.1	7	MS
29	Upahar	8	1	12.5	9	S
30	Jagabandhu	2	1	50	7	MS
31	IR 85086-SUB 33-3-2-1	18	18	100	1	T
32	IR 87092-26-3-1-3	30	12	40	9	S
33	IR 87098-55-2-1	20	16	80	5	M T
34	IR 87118-39-1-1-6	16	13	81.3	5	M T
35	IR 86256-6-2-2-2	13	13	0	9	S
36	IR 87439-BTN-88-3	13	7	53.9	7	MS
37	IR 87439-BTN-145-2-1	14	14	100	1	T
38	IR 88228-33-3-5-2	5	5	100	1	T
39	IR 88230-60-1-2-2	20	4	20	9	S
40	IR 88234-STG 11-1-1-1	5	5	100	1	T
41	IR 88243-17-1-1-3	20	6	30	9	S
42	IR 88250-20-1-1-3	21	21	100	1	T
43	IR 88760-SUB 93-3-3-3	15	4	26.7	9	S
44	IR 88762-SUB 51-3-1-3	19	13	68.4	7	MS
45	IR 88763-SUB 177-1-1-2	23	6	20.1	9	S
46	IR 88764-SUB 30-1-1-2	20	20	100	1	T
47	IR 88776-SUB 8-1-1-2	17	17	100	1	T
48	IR 88789-SUB 64-2-2-3	2	1	50	7	MS
49	IR 89246-SUB 38-3-2-1	2	0	0	9	S
50	IR 89262-SUB 5-2-3-2	3	0	0	9	S
51	Lalat	10	0	0	9	S

showed survival of 100% with a SES score of 1 which may be either due to submergence tolerance or have escaped as elongating type. The cultivars showing high percentage of survival were Savitri-Sub 1, IR 64-Sub

Fig.1. Screening for submergence tolerance under control conditions

1, PSBRc 18-Sub1, Swarna-Sub1, IR 85086-SUB 33-3-2-1, IR 87439-BTN-145-2-1, IR 88228-33-3-5-2, IR 88234-STG 11-1-1-1, IR 88250-20-1-1-3, IR 88764-SUB 30-1-1-2 and IR 88776-SUB 8-1-1-2. Six genotypes showed moderate tolerance reaction to submergence tolerance exhibiting 68.8 to 89.5% survival with a SES score of 5. The genotypes showing moderate tolerance reaction were Ciherang-Sub1, Thadokkham- Sub1, Inpara-3, IR 87098-55-2-1, IR 87118-39-1-1-6 and PSBRc 68. Twenty six genotypes were observed to be highly susceptible with a tiller survival of 0-26.7 % showing score of 9. While, seven genotypes behaved as moderately susceptible with survival of 50-68.4%. Considerable variation for survival

Table 3. Molecular markers used for genotyping of lowland rice genotypes for Sub1 gene cluster.

Sl. No.	Primer name	Oligonucleotide Primer sequence
1	RM8300 (F)	5' GCT AGT GCA GGG TTG ACA CA 3'
	RM8300 (R)	5' CTC TGG CCG TTT CAT GGT AT 3'
2	AEX (F)	5' AGG CGG AGC TAC GAG TAC CA 3'
	AEX (R)	5' GCA GAG CGG CTG CGA 3'
3	Sub 1 A203 (F)	5' CTT CTT GCT CAA CGA CAA CG 3'
	Sub 1 A203 (R)	5' AGG CTC CAG ATG TCC ATG TC 3'
4	Sub 1 BC 2 (F)	5' AAA ACA ATG GTT CCA TAC GAG AC 3'
	Sub 1 BC 2 (R)	5' GCC TAT CAA TGC GTG CTC TT 3'
5	Sub 1 C173 (F)	5' AAC GCC AAG ACC AAC TTC C 3'
	Sub 1 C173 (R)	5' AGG AGG CTG TCC ATC AGG T 3'

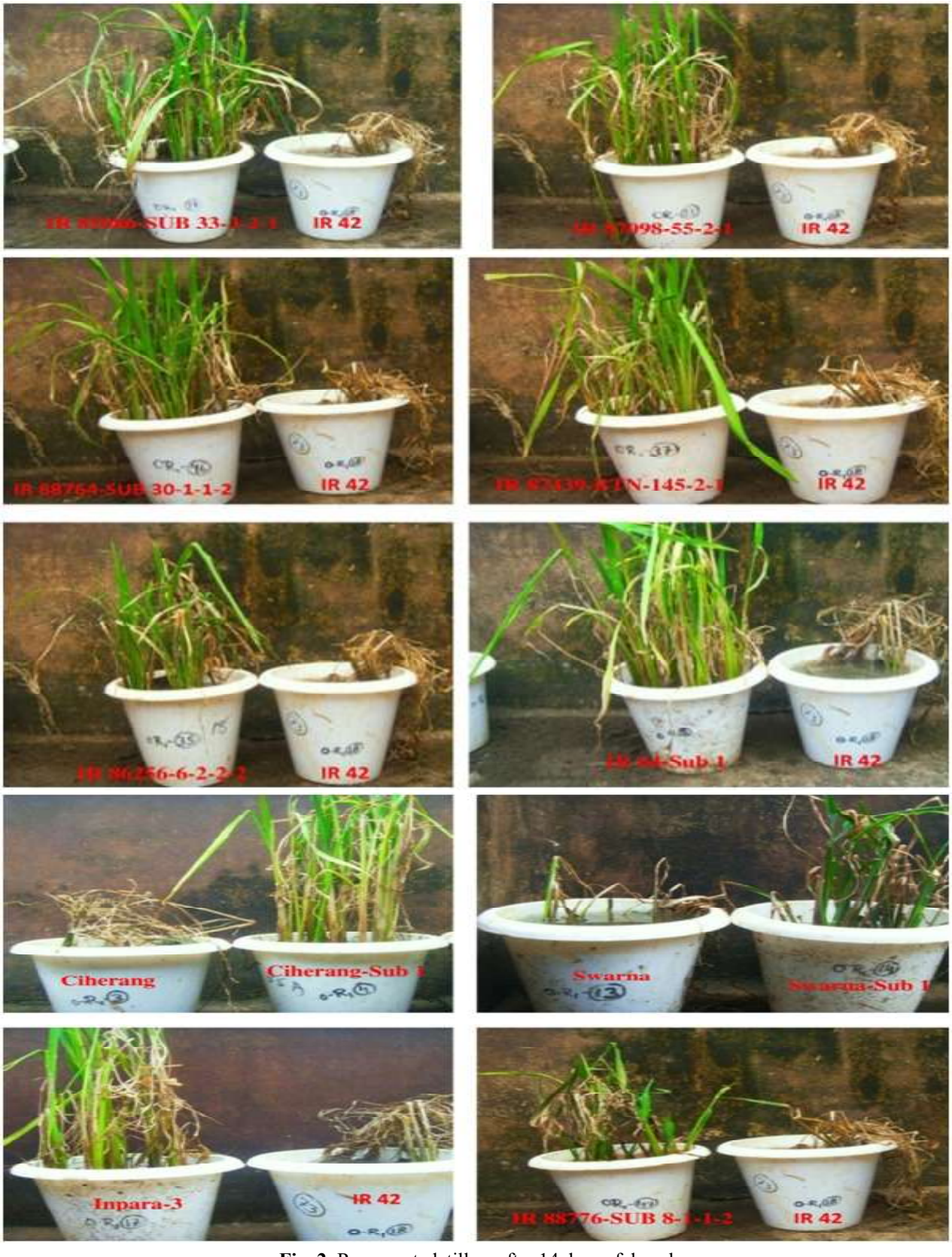

Fig. 2. Regenerated tillers after 14 days of de-submergence

was observed in the studied genotypes. The genotypes with high percentage of plant survival during submergence tolerance phenotyping are to be taken as submergence tolerant type. Hence, the Savitri-Sub1, IR 64-Sub1, PSBRc 18-Sub1, Swarna-Sub1, IR 85086-SUB 33-3-2-1, IR 87439-BTN-145-2-1, IR 88228-33-3-5-2, IR 88234-STG 11-1-1-1, IR 88250-20-1-1-3, IR 88764-SUB 30-1-1-2 and IR 88776-SUB 8-1-1-2 exhibited high submergence tolerance (score 1) may be used as cultivar or donor in the breeding programme. Genotypes with moderate tolerance to submergence coupled with low to moderate elongation ability may also have a breeding importance for lowland ecology. Five markers (Table 3) namely RM8300, AEX, Sub1A203, Sub1BC2 and Sub1C173 were used to screen the 51 lowland genotypes for *Sub1* gene cluster. The discrimination ability of the markers, either singly or in combination, for submergence tolerance was determined by clustering the genotypes by constructing the dendrogram on the basis of amplification pattern of the genotypes with the markers. Genotypes were classified into 10 sub- clusters. Cluster 3 was the biggest cluster with three sub-clusters. This cluster accommodates 22 genotypes of which most of the genotypes were tolerant and moderately tolerant type with inclusion few susceptible types. The cluster 2 accommodated all the tolerant, moderately tolerant and moderately susceptible genotypes. Cluster1 accommodated all the highly and few moderately susceptible genotypes in it. Cluster 1 was the smallest cluster with 15 genotypes in it and all the genotypes are susceptible to submergence tolerance. Cluster 2 accommodated 14 genotypes majority of which are susceptible type with inclusion of very few tolerant and moderately tolerant type genotypes. It was hypothesized that the marker or combination of markers that can group the tolerant and susceptible genotypes into different cluster should be considered to be the best marker or marker combination. In this study, amplification for at least one direct marker and one bracket pair is presumed to be submergence tolerant genotype. Here, the pair marker can be taken as RM 8300 with Sub1BC2 or Sub1C173. Sub1A203, a direct marker for submergence tolerance could screen better as compared to markers like AEX, Sub1BC2 and RM 8300. AEX is a specific designed DNA marker with SNP at its 3' end and was exclusively designed for rice genotype IR40931containg *Sub1A* allele (Septiningsih

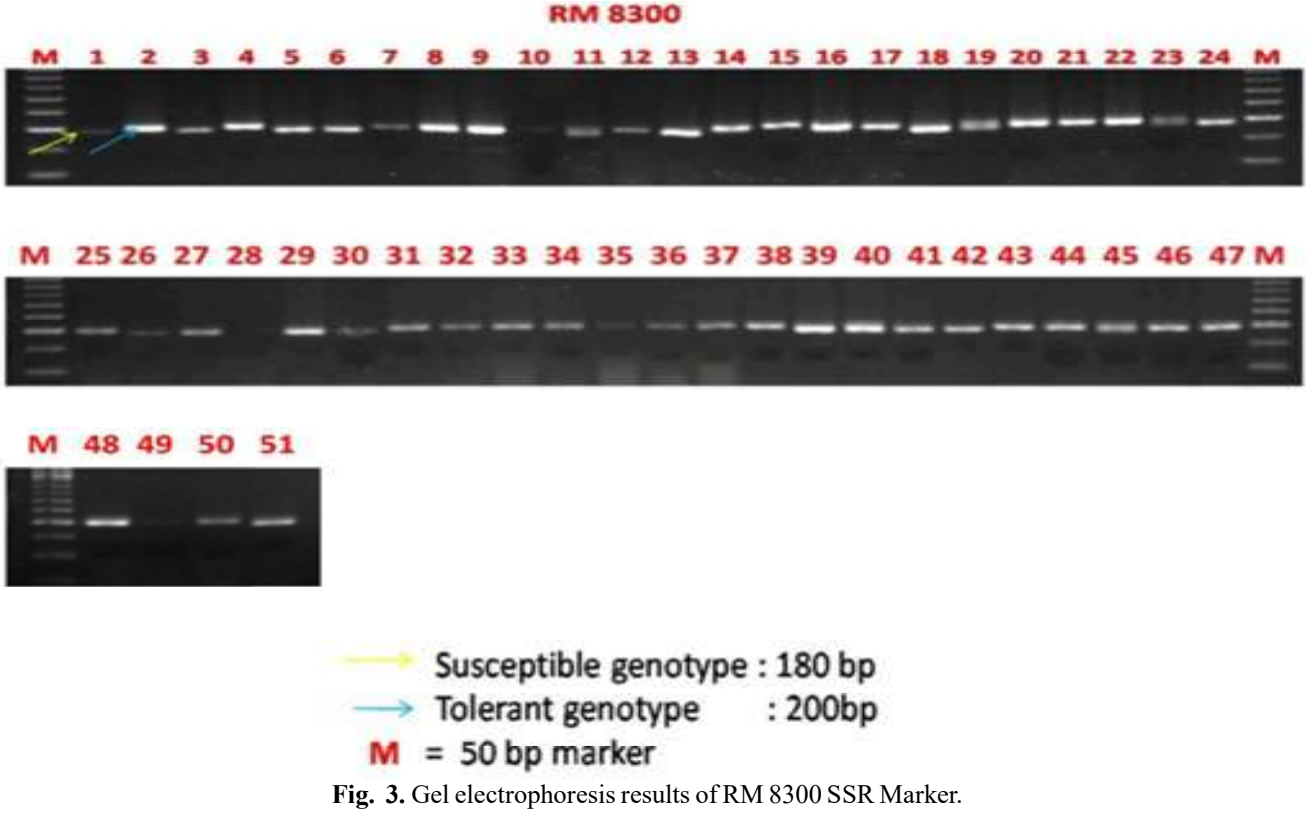

Fig. 3. Gel electrophoresis results of RM 8300 SSR Marker.

et al., 2009). Hence, this marker may not be able to differentiate other genotypes, which is reflected from the present study. The linked microsatellite marker RM8300 is located 300kb away from *Sub1A* allele. So, there is chance that this marker may not always be able to differentiate the genotypes perfectly for the presence of *Sub1A* locus. However, this marker could differentiate majority of the tolerant genotypes as observed under phenotyping for submergence tolerance. Sub1BC2 marker being an intergenic marker of Sub1B and Sub1C stood next to Sub1A203 in terms of discrimination ability which is quite obvious. However, some susceptible types were grouped into tolerant clusters.

Accordingly, 27 genotypes namely BR 11-Sub 1, Savitri-Sub1, IR 64-Sub1, Samba Mahsuri-Sub1, Swarna-Sub1, Thadokkham-Sub1, Ciherang-Sub1, Inpara-3, PSBRc 68, PSRBc 18, NSICRc 214, IR 85086-SUB 33-3-2-1, IR 87098-55-2-1, IR 86256-6-2-2-2, IR 87439-BTN-88-3, IR 87439-BTN-145-2-1, IR 88228-33-3-5-2, IR 87092-26-3-1-3, IR 88760-SUB 93-3-3-3, IR 88762-SUB 51-3-1-3, IR 88763-SUB 177-1-1-2, IR 88764-SUB 30-1-1-2, IR 88776-SUB 8-1-1-2, IR 88789-SUB 64-2-2-3, IR 89246-SUB 38-3-2-1, Thadokkham and IR 89262-SUB 5-2-3-2 exhibited five or four resistance bands (Fig. 4). Hence, it may be presumed that these genotypes may contain Sub1A allele. However, genotype like IR 68, Mahanadi, IR 72, IR 74 and IR 87118-39-1-1-6 showed three resistance bands (Fig. 4). Hence, these genotypes may or may not contain Sub1A allele. Genotypes like Sambha Mahsuri, Swarna, IR 42, IR 64, BR 11, Ciherang, Savitri, PSBRc102, OR-142-99, Pratikshya, Uphar, Jagabandhu, IR 88230-60-1-2-2, IR88234-STG 11-1-1-1 and IR 88243-17-1-1-3 showed presence of at least one susceptible band for a direct marker and positive for a pair of flanking markers (Fig. 4) and hence these genotypes may not possess *Sub1A* allele. The occurrences of flash flood is a common feature along with frequent inundations for more than 2 weeks and may remain up to one month with a water depth of 30 to 50 cm. Similar conditions have been described by many scientists (Khush, 1984; Laffite et al., 2006; Sarkar et al., 2006; Sarkar and Bhattacharjee, 2011) where in they have described that some rice growing areas are affected by only flash flood or both flash flood and stagnant flooding in different times or years.

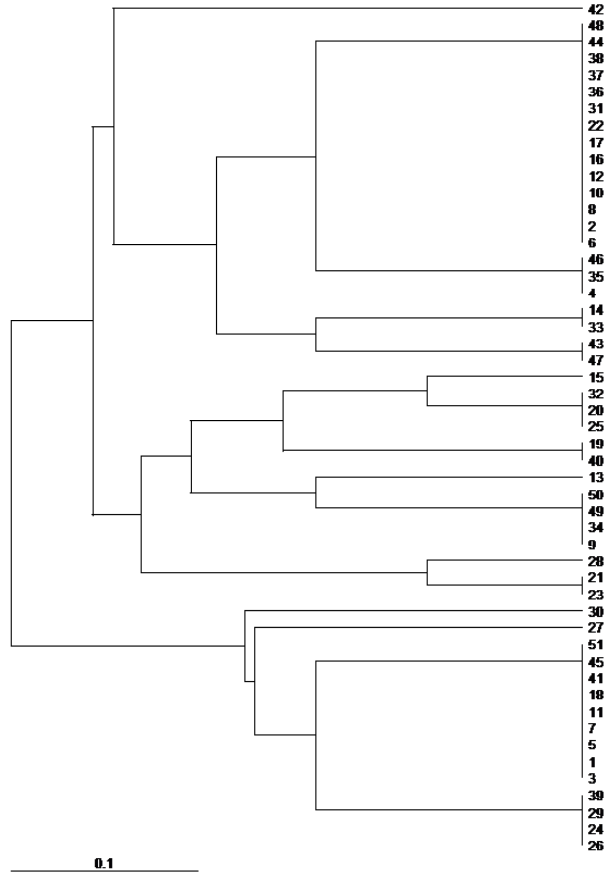

Fig. 4. Dendrogram showing 51 lowland rice genotypes with five Sub1 related markers.

Genotyping results using *Sub1* related molecular markers indicated that these genotypes possessed Sub1 allele. Hence, natural introgression of submergence gene may be the reason of tolerance in these moderately tolerant genotypes. These two traits should be in the breeding objectives of rainfed shallow lowland ecology. Standing flood with more than 25cm water depth may adversely affect growth and survival of modern varieties. It hinders in tillering and increases lodging and in some cases severe reduction in crop stands (Tuong et al., 2000; Singh et al., 2011; Ismail et al., 2013). Due to lack of high yielding varieties with these two traits, farmers of eastern region are also cultivating low yielding landraces possessing moderate elongation ability with submergence tolerance. In the study, six genotypes were found to possess moderate tolerance to submergence. Genotyping results also confirmed through the banding pattern (Fig. 4).These are also evidenced from the cluster analysis where one major cluster possessed higher proportion of genotypes with

submergence tolerance. Hence, this is a common adaptive feature seen in case of lowland genotypes of eastern India. Submergence tolerance in these genotypes may be due to *Sub1A* locus. Earlier reports (Xu and Mackill, 1996; Nandi et al., 1997; Toojinda et al., 2003) have already stated the role of *Sub1A* allele. When these tolerant genotypes were analyzed using Su1BC2 marker which is linked to *Sub1B* locus, it was observed that majority of the genotypes exhibited specific amplicon. This indicated a trend of negative association of *Sub1BC2* with submergence tolerance. When all susceptible and tolerant genotypes were analyzed, it revealed no association with *Sub1C* specific bands indicating that role of *Sub1C* may be ignored for submergence tolerance. It was noted earlier that limited expression of *Sub1C* was associated with tolerance (Xu et al, 2006). But, Septiningsih et al., 2009 reported non-significant contribution by *Sub1C* allele. The highly tolerant genotypes for submergence such as Savitri-Sub 1, IR 64-Sub1, PSBRc 18-Sub1, Swarna-Sub1, IR 85086-SUB 33-3-2-1, IR 87439-BTN-145-2-1, IR 88228-33-3-5-2, IR 88234-STG 11-1-1-1, IR 88250-20-1-1-3, IR 88764-SUB 30-1-1-2 and IR 88776-SUB 8-1-1-2 may be evaluated for cultivar or donor parent for flash flood areas.

CONCLUSION

The materials under the present investigation possess a highly significant difference among the test for all the traits, indicating presence of substantial genetic variation and thus provide enough scope for effective selection. The screening of genotypes for submergence tolerance under control facility revealed that the cultivars like Savitri-Sub1, IR 64-Sub1, PSBRc 18-Sub 1,Swarna-Sub1, IR 85086-SUB 33-3-2-1, IR 87439-BTN-145-2-1, IR 88228-33-3-5-2, IR 88234-STG 11-1-1-1, IR 88250-20-1-1-3, IR 88764-SUB 30-1-1-2 and IR 88776-SUB 8-1-1-2 were identified as tolerant to submergence with high percentage of survival (100% with a SES score of 1), Similarly, the genotypes showing moderate tolerance reaction (68.8 to 89.5% survival with a SES score of 5) were Ciherang-Sub1, Thadokkham-Sub1, Inpara-3, IR 87098-55-2-1, IR 87118-39-1-1-6 and PSBRc 68 and seven genotypes with moderate tolerance to submergence coupled with low to moderate elongation ability may also have a breeding importance for lowland ecology. The discrimination ability of the markers, either singly or in combination, for submergence tolerance was determined by clustering the genotypes by constructing the dendrogram on the basis of amplification pattern of the genotypes with the markers. Genotypes were classified into three cluster and 10 sub- clusters. Cluster 3 was the biggest cluster with three sub-clusters. . By taking into account the phenotyping and genotyping results, cultivars like Savitri-Sub 1, IR 64-Sub1, PSBRc 18-Sub1, Swarna-Sub1, IR 85086-SUB 33-3-2-1, IR 87439-BTN-145-2-1, IR 88228-33-3-5-2, IR 88234-STG 11-1-1-1, IR 88250-20-1-1-3, IR 88764-SUB 30-1-1-2 and IR 88776-SUB 8-1-1-2 were identified as tolerant to submergence and may be used as cultivar or donors in the breeding programme.

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