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Submergence Tolerant Androgenic Rice Dihaploids Derived from Intra- and Interspecific Crosses: I. Seed Storage Protein and Isozyme Pattern Through Gel Electrophoresis

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Abstract

Two cultivated *indica* rice Pankaj and Mahsuri susceptible to submergence were crossed with submergence tolerant semi wild FR-13A and wild *O. rufipogon*. FR-13A had better submergence tolerance than *rufipogon* as revealed by submergence tolerance score. F_1s of *rufipogon* exhibited higher cell abnormality in meiotic divisions and recorded high pollen sterility. F_1s of Pankaj x FR-13A and Pankaj x *rufipogon* exhibited higher level of submergence tolerance indicating crosses involving Pankaj with either FR-13A or *rufipogon* appeared to be better cross combinations. This would suggest that gene/s responsible for submergence tolerance are expressed more efficiently against the genetic background of Pankaj. Androgenic dihaploids were raised through anther culture from F_1s. Submergence tolerant androgenic dihaploid lines were identified through two passages of submergence tolerant screening test. They were evaluated for their seed protein polymorphism through SDS-PAGE as well for their esterase and peroxidase banding patterns. A general trend became evident where lower molecular wieght bands of either esterase or peroxidase as well seed protein similar to FR-13A and *rufipogon* were observed in submergence tolerant androgenic dihaploid lines. The dihaploid lines derived either from the crosses between Pankaj and FR-13A or between Pankaj and *rufipogon* displayed better survivability under submergence. Our results, therefore indicate that submergence tolerance favours the genetic background of Pankaj for its expression where FR-13A proves to be desirable donor for submergence trait.

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Introduction

Identification and development of submergence tolerant rice genotypes is a pressing international problem in rice improvement programme. This is particularly of great concern in West Bengal where water inundation during rice cultivation is a very frequent and common experience. The development of submergence tolerant genotypes of rice is therefore of great practical importance. Several groups of scientists have been working on development of submergent tolerant rice (Mackill et al. 1993, Mandal et al. 1995, Mandal and Gupta 1997, 1999). However, such attempts are yet to deliver suitable cultivars which can profitably be utilized under the prevailing conditions of flash-floods in West Bengal. To address this problem in a meaningful manner the present study was undertaken to develop submergence tolerant rice genotypes with the help of anther culture to speed the breeding process and isolate unusual recombinant. Promising rice genotypes were therefore crossed with available submergence tolerant semi wild type *indica* (FR-13A) as well with wild (*O. rufipogon*) to transfer gene(s) conferring tolerance to submergence. Subsequently, androgenic dihaploids were raised through anther culture, in order to produce homozygous lines of submergence tolerant gene(s). The developed lines were studied for seed storage protein profile as well as esterase and peroxidase isozyme patterns through gel electrophoresis. Submergent tolerant progeny, showing isozyme profile with tolerant parents could indicate co-segregation of particular isozyme with the tolerant trait.

Materials and Methods

Two promising widely cultivated *indica* rice genotypes Pankaj and Mahsuri, susceptible to submergence were used in the crossing programme. They were corssed with submergence tolerant FR-13A (semi wild) and *O. rufipogon* (wild). F_1s derived were cytologically examined to record the fertility level and cell abnormality during meiotic division. F_1s were tested for their submergence tolerance after complete submergence of ten-day-old seedling for a period of ten days. The submergence tolernce of F_1s was scored following the scoring schedule as proposed by International Rice Research Institure (IRRI), Philippines (Vergara and Mazaredo 1975). It was suggested that lower the score the higher was the tolerance. The degrees of tolerance of the F_1s were compared with their respective parents referred to as control.

The anthers at the uninucleate microspore stage were collected at 8 to 9 a.m. from the selected submergence tolerant lines and androgenic dihaploids were raised as described earlier (Mandal and Gupta 1995a). Only green

regenerant dihaploids were cytologically examined following the procedure of Mandal and Gupta (1995b). Healthy normal looking dihaploid seedlings were transplanted and normal cultural practices were followed as done in rice cultivation to raise the dihaploids. Seeds collected from these were sown to raise the A_2 generation in pots. Ten-day-old seedlings were screened following submergence for a period of ten days. The survived seedligns were allowed to grow for another 32 days. They were again submerged for ten days. Following these two steps rigorous screening for submergence the survival lines were raised to maturity. From the harvested seeds (A_3 generation) seed protein profile along with their parents were analysed and simultaneously ten-day-old seedlings raised from them were subjected to peroxidase and esterase isozyme analysis.

Seed protein content of genotypes was estimated following Lowry et al. (1951). To study seed protein polymorphism, one dimensional SDS-PAGE (15% separating gel and 4% stacking gel) was carried out following Laemmli (1970) in a regular vertical gel system (BIOTECH). For this purpose total protein was extracted after suspending seed flour for 45 min in an extraction buffer (Tris-HCl, pH 6.8), followed by centrifugation at 10,000 rpm at 4_C for 20 min. Then the protein sample along with sample buffer was heated for 3 - 5 min in boiling water bath and gradually cooled 100 µg protein was loaded in sample well. Molecular weight marker was also incorporated into the gel to determine the molecular weight of the bands. The gel was run at 40 mA for 3 h followed by staining in Coomassie Brilliant Blue R250 overnight.

For the study of isozymes esterase and peroxidase, native PAGE was performed with 7.5% gel. Electrophoresis of esterase was done according to the method of Kehlar and Allard (1970). For this purpose 0.5 g of fresh leaf sample was macerated in tris-citric acid buffer (pH 7.8) at 4_C. The homogenates were centrifuged at 10,000 rpm for 30 min at 0_C. Fifty µg protein was loaded in each lane and the gel was run at 20.4 mA for 2 h. The gel was stained using Fast Blue RR Salt and α-naphthyl acetate. For the study of peroxidase also 0.5 g leaf sample was macerated in phosphate buffer (pH 7.0) at 4_C followed by centrifugation for 30 min. Staining was done according to the method proposed by Welter and Dyck (1983) using α-dianisidine. Percentage dissimilarity was calculated from the presence and absence of bands according to the method propsed by Whitney et al. (1968).

Results and Discussion

F_1s-seed setting, cell abnormality and submergence tolerance: In the crosses involving Pankaj x FR-13A and Mahsuri x FR-13A better seed setting percentage was observed as compared to their crosses with *rufipogon* (Table 1). This is expected as *rufipogon* is a wild species which exhibited higher order of cell abnormalities during meiotic divisions where per cent filled grain was as low as 17. There was a close correspondence (r = 0.93) between pollen sterility per cent filled grains. The degree of submergence tolerance was conspicuously different between FR-13A and *rufipogon* where the former showing at least 1.5 times more tolerance to submergence than the latter as lower score value was

Table 1. Submergence tolerance, pollen sterility, cell abnormality along with filled grain percentage of parents and their hybrids.

Genotype	Tolerance* (1 - 10 scale)	Pollen sterility (%)	Filled grain (%)	Cell abnormality during meiosis (%)
Pankaj (P)	7.51	8	95	Negligible
Mahsuri (M)	8.48	6	92	"
FR-13A (F)	2.24	9	94	"
O. rufipogon (R)	3.10	37	17	56
P x F	1.77	28	77	21
P x R	2.01	35	62	46
M x F	2.62	27	71	18
M x R	2.52	33	69	36

*Lower the score is higher the toerance.

the index of higher tolerance (Vergara and Mazaredo 1975). However, F_1s of Pankaj x FR-13A and Pankaj x *rufipogon* showed higher level of submergence tolerance as compared to F_1s derived from the corsses involving Mahsuri. Interestingly, F_1 of the Pankaj x FR-13A exhibited highest tolerance to submergence, which was even better than the FR-13A, the donor parent. Similarly, F_1 of Pankaj x *rufipogon* was better than *rufipogon* itself for submergence tolerance. In other words, crosses involving Pankaj appeared to be the better corss combinations with either of the FR-13A or *rufipogon* in exhibiting high level of tolerance. This would suggest that gene(s) of submergence tolerance were able to express more efficiently against the genetic background of Pankaj.

Seed protein: Seed storage protein profiling through SDS-PAGE showed differences in total number of bands for the two susceptible genotypes, Pankaj

and Mahsuri and for two submergence tolerant semi-wild FR-13A and wild *O. rufipogon*. The protein bands were arbitrarily grouped as high, intermediate and low molecular weight groups corresponding to more than 100 kD, 30 to 100 kD and less than 30 kD, respectively. As many as six bands were grouped as high molecular weight which were commonly found in either susceptible or tolerant parents was well their dihaploids. With respect to intermediate and low molecular weight groups Pankaj differed from Mahsuri and in each case Pankaj had one band less than that of Mahsuri. In the case of submergence tolerant FR-13A and *O. rufipogon* difference was also in the number of intermediate molecular weight group bands. FR-13A had 12 bands but *O. rufipogon* exhibited only 11 (Table 2). Dihaploids derived from either Pankaj x FR-13A or Pankaj x *rufipogon* displayed similar band profile belonging to these three molecular weight groups as observed in FR-13A or in *rufipogon* (Figs. 1a and 1b). Similar band pattern was observed in the dihaploids derived from the corss, Mahsuri x FR-13A or Mahsuri x *rufipogon*. It can be suggested that selected submergence tolerant dihaploids continued to maintain the banding pattern of either FR-13A or *rufipogon*.

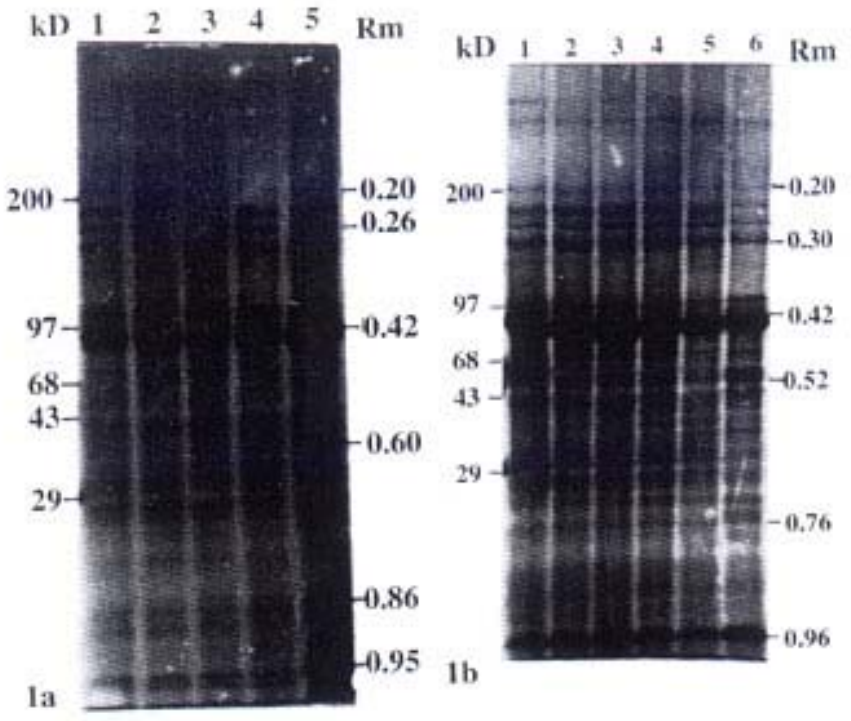
Table 2. SDS-PAGE seed protein bands under different MW groups for parents and their androgenic dihaploids.

Genotypes	HMW (> 100 kD)	IMW (30 - 100 kD)	LMW (< 30 kD)	Total No. of bands
Pankaj (P)	6	11	11	28
Mahsuri (M)	6	12	12	30
FR-13A (F)	6	12	12	30
O. rufipogon (R)	6	11	12	29
PF	6	12	12	30
PR	6	11	12	29
MF	6	12	12	30
MR	6	12	12	30

HMW = High mol. wt., IMW = Intermediate mol. wt., LMW = Low mol. wt. PF, PR, MF and MR are the androgenic dihaploids of respective hybrid combinations.

Peroxidase: The band profile of peroxidase recorded from androgenic dihaploids derived from Pankaj x FR-13A and Pankj x *rufipogon* as well as from Mahsuri x FR-13A and Mahsuri x *rufipogon* was studied and compared with their respective parents. Both Pankaj and Mahsuri exhibited nine bands but they differed in mobility. As opposed to this FR-13A had as many as eight peroxidase bands (Fig. 2a) while there were five in *rufipogon* (Fig. 2b) indicating distinct difference in band number and its mobility. High molecular

weight peroxidase bands did not differ. The difference was more conspicuous in intermediate and low molecular weight bands. Similarly, androgenic dihaploids derived from Pankaj x FR-13A displayed banding pattern of FR-13A and FR-13A is knwon to have better submergence tolerance than that of *O. rufipogon* (Table 1). In other words peroxidase genes from FR-13A seem to have transferred to the Pankaj genetic background in a stable manner. Such transfer of gene did not occurr in case of crosses, Mahsuri x *rufipogon*. This is supported from the analysis of dissimilarity percentage (Table 3A).

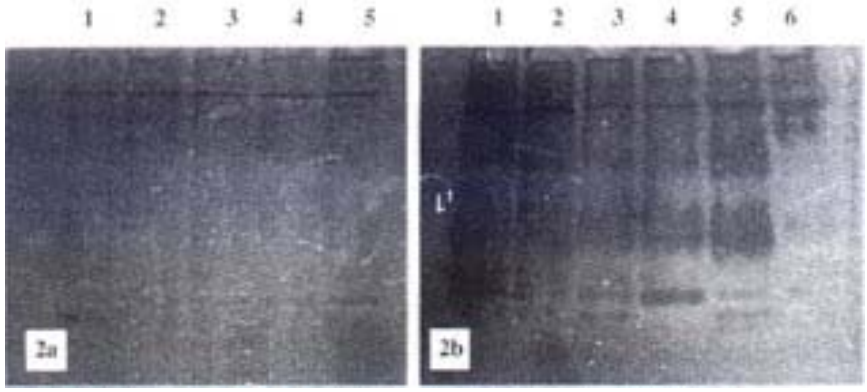


Figs. 1a - b: a. SDS-PAGE electrophoregram of seed storage protein in Mahsuri, FR-13A and their androgenic dihaploids. Lanes 1 to 3 dihaploids (MF1, MR2 and MF3), 4 and 5 represent Mahsuri and FR-13A, respectively. b. SDS-PAGE electrophoregram of seed storage protein in Pankaj, *rufipogon* and their androgenic dihaploids. Lanes 1 to 4 dihaploids (PR1, PR2, PR3 and PR4), 5 and 6 represent Pankaj and *rufipogon*, respectively.

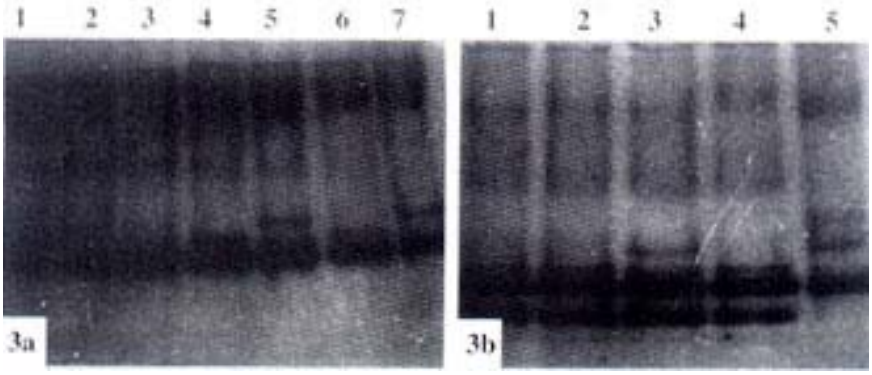
Exterase : As many as six and nine bands were observed in Pankaj and Mahsuri, respectively (Figs. 3a and 3b). There were no differences in band with high molecular weight. Bands of lower molecular weight in general were absent in Pankaj in comparison to Mahsuri. On the other hand, bands of both FR-13A and *rufipogon* have six bands each but they showed different mobility.

Such differences may not be a mere coincidence, as esterase has been previously shown to be a useful induction of abiotic stress tolerance (Zhang et al. 1988).

The dihaploids of Pankaj x *rufipogon* in general maintained the banding pattern of either FR-13A or *rufipogon*. Similar was the case in the dihaploids of Mahsuri x FR-13A and Mahsuri x *rufipogon*. However, two lines PF1 and PF4 of Pankaj x FR-13A and a dihaploid line MR1 or Mahsuri x *rifipogon* displayed a distinct departure in banding pattern though they were submergence tolerant (Table 3B).



Figs. 2a - b: a. Banding pattern of peroxidase in Mahsuri, FR-13A and their androgenic dihaploids. Lanes 1 - 3 dihaploids (MF1, MF2 and MR3), 4 and 5 represent Mahsuri and FR-13A, respectively. b. Banding pattern of peroxidase in Pankaj, *rufipogon* and their androgenic dihaploids. Lanes 1 to 4 dihaploids (PR1, PR2, PR3 and PR4), 5 and 6 represent Pankaj and *rufipogon*, respectively.



Figs. 3a - b: a. Banding pattern of esterase in Pankaj, FR-13A and their androgenic dihaploids. Lanes 1 to 5 dihaploids (PF1, PF2, PF3, PF4 and PF5), 6 and 7 represent Pankaj and FR-13A, respectively. b. Banding pattern of esterase in Mahsuri, FR-13A and their androgenic dihaploids. Lanes 1 to 3 dihaploids (MF1, MF2 and MF3), 4 and 5 represent Mahsuri and FR-13A, respectively.

Table 3A. Dissimilarity percentage calculated from peroxidase banding pattern of androgenic dihaploids and their parents.

	PF1	PF2	PF3	PF4	PF5	P	F
PF1	0	0	0	0	0	11.11	0
PF2		0	0	0	0	11.11	0
PF3			0	0	0	11.11	0
PF4				0	0	11.11	0
PF5					0	11.11	0
P						0	11.11
F							0
	PR1	PR2	PR3	PR4	P	R	
PR1	0	0	0	11.11	20.00	44.44	
PR2		0	0	11.11	20.00	44.44	
PR3			0	11.11	20.00	44.44	
PR4				0	42.86	37.50	
P					0	44.44	
R						0	
	MF1	MF2	MF3	M	F		
MF1	0	20.00	20.00	20.00	45.45		
MF2		0	0	0	45.45		
MF3			0	0	45.45		
M				0	45.45		
F					0		
	MR1	MR2	MR3	MR4	MR5	M	F
MR1	0	10.00	0	10.00	10.00	10.00	50.00
MR2		0	10.00	0	0	0	44.44
MR3			0	10.00	10.00	10.00	50.00
MR4				0	0	0	44.44
MR5					0	0	44.44
M						0	44.44
R							0

P = Pankaj, M = Mahsuri, R = *rufipogon*; PF1-PF5, PR1-PR4, MF1-MF3 and MR1-MR5 = Dihaploids derived from the respective cross combinations.

Therefore, it can be correlated that lower molecualr weight bands of either esterase or peroxidase as well seed proteins in the submergence tolerant dihaploids may be similar to those of FR-13A and *rufipogon*. The complex nature of genetic system for submergence tolerance in rice can very well be projected if the survivability under submergence stress of these individual dihaploid lines is scored.

Table 3B. Dissimilarity percentage calculated from esterase banding pattern of androgenic dihaploids and their parents.

	PF1	PF2	PF3	PF4	PF5	P	F
PF1	0	14.29	14.29	0	14.29	0	50.00
PF2		0	0	14.29	0	14.29	60.00
PF3			0	14.29	0	14.29	60.00
PF4				0	14.29	0	50.00
PF5					0	14.29	60.00
P						0	50.00
F							0
	PR1	PR2	PR3	PR4	P	R	
PR1	0	12.50	12.50	12.50	44.44	14.29	
PR2		0	0	0	55.56	37.50	
PR3			0	0	55.56	37.50	
PR4				0	55.56	37.50	
P					0	66.67	
R						0	
	MF1	MF2	MF3	M	F		
MF1	0	0	0	10.00	54.55		
MF2		0	0	10.00	54.55		
MF3			0	10.00	54.55		
M				0	63.64		
F					0		
	MR1	MR2	MR3	MR4	MR5	M	R
MR1	0	0	0	0	0	0	33.33
MR2		0	0	0	0	0	33.33
MR3			0	0	0	0	33.33
MR4				0	0	0	33.33
MR5					0	0	33.33
M						0	33.33
F							0

P = Pankaj, M = Mahsuri, R = *rufipogon*, PF1-PF5, PR1-PR4, MF1-MF3 and MR1-MR5 = Dihaploids derived from the respective cross combinations.

Table 4. Survivability (%) under submergence at seedling stage (four-week-old).

Parents		Androgenic dihaploids derived from the crosses of							
Names	%	Pankaj ∇ FR-13A		Mahsuri ∇ FR-13A		Pankaj ∇ *rufipogon*		Mahsuri ∇ *rufipogon*	
		Lines	%	Lines	%	Lines	%	Lines	%
Pankaj	51.4	PF1	88.6	MF1	78.8	PR1	83.8	MR1	85.4
Mahsuri	32.7	PF2	82.9	MF2	86.8	PR2	81.5	MR2	80.6
FR-13A	90.0	PF3	89.6	MF3	77.5	PR3	87.6	MR3	69.3
Rufipogon	91.6	PF4	81.4			PR4	88.2	MR4	63.2
		PF5	80.8					MR5	79.4

Survivability per cent at seedling stage (three-week-old) under sub-mergence : The study by and large demonstrated a general trend where dihaploid lines derived from either the crosses between Pankaj and FR-13A or Pankaj x *rufipogon* displayed better survivability (Table 4). In other words, against the genetic background of Pankaj the submergence tolerance finds better expression. In this scheme of theme FR-13A appeared to be better donor for submergence tolerance in rice. It is, therefore, reasonable to suggest that to develop submergence tolerance genotypes appropriate matching genotype (in this case Pankaj) needs to be identified and androgenic dihaploids according to the testing procedures outlined would provide meaningful outcome.

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Table 4. Survivability (%) under submergence at seedling stage (four-week-old).

Parents		Androgenic dihaploids derived from the crosses of							
Names	%	Pankaj ∇ FR-13A		Mahsuri ∇ FR-13A		Pankaj ∇ *rufipogon*		Mahsuri ∇ *rufipogon*	
		Lines	%	Lines	%	Lines	%	Lines	%
Pankaj	51.4	PF1	88.6	MF1	78.8	PR1	83.8	MR1	85.4
Mahsuri	32.7	PF2	82.9	MF2	86.8	PR2	81.5	MR2	80.6
FR-13A	90.0	PF3	89.6	MF3	77.5	PR3	87.6	MR3	69.3
Rufipogon	91.6	PF4	81.4			PR4	88.2	MR4	63.2
		PF5	80.8					MR5	79.4

(1a) (1b) (1c) (1d)

(2a) (2b)

(1a) (1b) (1c) (1d)

(2a) (2b)