Identification of *Fusarium* isolates for use in pathogen derived resistance in the management of seed discolouration of rice

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ABSTRACT

Management of Fusarium species associated with seed discolouration of rice was attempted through pathogen derived resistance (PDR). Twenty Fusarium isolates associated with rice varieties Lunishree, Pooja and Sarala were classified into six colour groups on the basis of their colour on Potato Dextrose Agar media. The variability in cultural, molecular characters and virulence were observed among the cultures, associated with the samples of a cultivar drawn from the same seed lot. The vigour index of seedlings of rice cultivar Utkalprabha and Savitri, treated with isolate F14A was considerably more than the untreated control. RAPD-PCR of twelve isolates was done using two primers, AP12h and R-108. The isolate F14A was found to be a fast growing isolate, distinctly different from other isolates and may be suitable for management of this devastating disease by Pathogen Derived Resistance.

Key words: *Fusarium, discolouration, rice, pathogen derived resistance*

Seed discolouration has emerged to be a major constraint in cultivating the photo-sensitive high yielding rice cultivars during the rainy season. *Fusarium* was found to be associated with seed discolouration in long duration high yielding rice varieties and yield losses up to 20% were recorded due to this pathogen in the coastal region of Orissa. The fungus infects plants through roots or crowns later becomes systemic and infects the panicle. The infected spiklets are unfilled or partially filled. The microconidia and mycelium of the pathogen are found to be concentrated in the vascular bundles. Significant genetic variability was observed in the pathogen collected from the coastal region of Orissa (Mohanty and Dhua, 2007). The host plant resistance and chemical control measures are not very dependable against this pathogen. Under these circumstances, the pathogen derived resistance (PDR) which has been evolved from cross protection could be a better option. In cross protection a mild or attenuated strain of a pathogen is used to protect crops from infection by a virulent strain of a related organism.

Application of molecular techniques has provided methods for identification of isolates of plant pathogens. Molecular methods were found to be useful for distinguishing the non-taxonomic categories e.g. virulence and toxicity. The virulence within a race was identified in vitro by RAPD (Mes *et al.*, 1999 and Williams *et al*., 1990).

The present investigation was carried out to study the cultural, virulence and molecular characters of rice seed borne *Fusarium isolates*, which may be used for cross protection against infection by virulent strain of this organism.

MATERIALS AND METHODS

Fusarium species were isolated from seeds of three rice cultivars such as Lunishree, Pooja and Sarala, by Blotter plate method (Agarwal and Sinclair, 2000). Seeds were first surface sterilized with 30% alcohol (for 30seconds), rinsed twice with sterile water and then plated on sterilized blotting paper, soaked with sterilized water. After 4-5 days when the micro-flora were visible, *Fusarium spp* were isolated on Potato Dextrose Agar (PDA-Peeled Potato-250g, Dextrose-20g, Agar-15g and pH-5.6) and stored at 4^0C on PDA slants. Pigmentations of isolates were studied by growing on PDA slants and comparing with R.H.S. Colour Chart

(The Royal Horticultural Society, London: Flower Council of Holland, Leiden). Isolates were grown on potato dextrose broth for 6 days in still culture and fresh weight of mycelial mat was recorded.

The seeds of rice varieties Savitri and Utkalprabha were soaked overnight in sterilized water. After Surface sterilization first with 70% Ethanol and sodium hypochlorite solution, the seeds were exposed to *Fusarium* inoculum by keeping them on ten days old *Fusarium* cultures, in culture plates, for four days. Treated and untreated seedlings were then transferred to the moist blotting paper for observations. Seedling vigor was determined by the following formula: Vigor Index (VI) = Germination Percentage × (Shoot length + Root length). The fresh root weight of germinated seeds from each isolate was estimated on the ninth day after inoculation on *Fusarium* culture plates.

DNA was isolated from fungal mycelia by following the protocol of Liu *et al*. (2000). To a lump of mycelia 1ml of extraction buffer **(**400 mM Tris HCL pH 8.0, 60mM EDTA pH 8.0, 150mM NaCl,1% SDS) was added and ground with glass rod and mortar until a fine solution was formed. After keeping for 15 minutes at room temperature, 100µl of potassium acetate was added, mixed and then centrifuged at 10000 rpm for 1 minute. To the supernatant 500µl of Chloroform: Isoamyl Alcohol (24:1) was added and then centrifuged for 10minutes at 10000 rpm. To the aqueous phase equal amount of isopropanol was added, and kept at -20^{0}C overnight. The solution was then centrifuged at 10000 rpm for 3 minutes and the pellet was washed with 70% ethanol twice, air dried and redissolved in 50µl of 1X TE buffer (pH 8). The quality and quantity of the extracted DNA was checked by agarose gel electrophoresis.

Amplification was done following Guerra *et al.* (2000) using two primers, AP12h (5'CGGCCCCTGT3') and R-108 (5'GTATTGCCCT3'). The 50µl reaction mixture contained 1X PCR buffer, 1.5mM $MgCl_2$, 10 pico moles of primer, 0.1mM dNTP and 1U of Taq DNA polymerase. Amplification was done in a Thermal cycler (PTC-100, M.J. Research. INC). with an initial denaturation at 93^{0}C for 3 minutes and then denaturation at 93^{0}C for one minute, annealing at 36^{0}C for one minute, extension at 72^{0}C for one minute and a final extension at 72^{0}C for 5 minute. Amplified products were size fractionated in 2% agarose gel and visualized by Ethidium bromide staining.

Each profile was compared on the basis of the presence (1) versus absence (0) of amplified band. The data generated from RAPD for each isolate were pooled. A genetic similarity matrix was calculated based on the method of simple matching coefficient and the values were used to generate a similarity matrix. The resulting matrix was analyzed for clustering of the isolates by the un-weighted pair-group with arithmetic averages (UPGMA). All calculations were conducted by using the computer program NTSYS 2pc (Rholf, 1990).

RESULTS AND DISCUSSIONS

Twenty *Fusarium* isolates were found to be associated with the long duration rice cultivars Pooja, Lunisree and Sarala at CRRI, farm. These isolates were grouped in to ten major colour groups, following the "R.H.S. Colour Chart numbers" (Table 1). Colour variability was observed among the cultures, isolated from the samples of cultivar drawn from the same seed lot.

Variability in seedling vigor was observed when exposed to *Fusarium* species. The vigour index of Utkalprabha seedlings, treated with *Fusarium* isolate number F14A was almost double than the untreated control. The vigour index of Savitri seedlings increased three times after treatment with F14A. Virulent isolates F55 and F55A caused seedling mortality in both the cultivars. Isolate F22, F45 and F47 considerably reduced the vigour of Savitri seedling whereas F36, F82, F90 and F91did not affect this cultivar. More than 50% reduction in vigor of Utkalprabha seedlings was observed by F36, F45 and F82 (Figure1). Roots of seedlings were damaged by F36, F47, F82, F45, F90, F91, and F22. Root weight of F14A treated seedlings was at par with untreated control (Figure 2).Maximum mycelial weight was observed in F22 followed by F91 and F14A (Table 2).

Amplification resulted in the generation of 122 fragments (Figure 3) between isolates out of which 95% were polymorphic as observed by Muller *et al.* (2005). Coefficient of similarity among Fusarium isolates ranged from 37-89% (Table 3). Dendrogram showed that the isolates can be grouped in to two major clusters (Figure 4). The first cluster consisted of eight isolates

Table 1. Passport data and Colour of *Fusarium* isolates

* Isolate ID	Isolated from rice variety	**Colour Group on PDA surface	**Colour No. on PDA bottom	PD broth colour	Colour of cultural filtrate
F91	Lunisree	Grey Red	179D	159A	19D
F81	Pooja	Grey Red	179D	76D	19D
F55	Sarala	Grey Red	186A	-	-
F76	Pooja	Orange	24C	80D	36A
F44	Sarala	Red	43D	38A	39A
F90	Sarala	Red	44B	63D	26D
F56	Sarala	Yellow	4D	32B	32C
F31	Sarala	Yellow	4D	158B	30D
F47	Sarala	Yellow	4D	25B	18A
F45	Sarala	Red purple	58A	39A	42B
F57	Sarala	Red purple	58A	35A	25C
F55A	Sarala	Red purple	59A	158A	11D
F52	Sarala	Red purple	59A	37A	11C
F14A	Pooja	Purple	76A	34A	32A
F72	Pooja	Purple	76B	69C	19D
F39	Sarala	Violet	83C	43B	39B
F82	Sarala	Violet	84A	45A	34A
F42	Sarala	Violet	84A	68D	37A
F22	Sarala	Violet	84A	33C	25C
F36	Sarala	Violet	84A	157C	30C

*CRRI, Plant Pathology culture collection **As per (Royal Horticultursl Society, London: Flower Council of Holland, Leiden) R.H.S. Colour Chart.

Table 2. Growth of *Fusarium* isolates on PD broth and their effect on root growth

Isolate I.D.	Mycelial wt.(gm.)	Utkalprabha Root wt **(mg)	Savitri Root wt ** (mg)
F22	4.777	12.5	10
F36	2.894	5	2.5
F14A	4.122	15	12.5
F44	2.883	7.5	7.5
F45	2.747	12.5	10
F47	3.422	10	7.5
F55	2.417	0	0
F55*	3.463	0	0
F82	3.814	10	7.5
F90	3.711	12.5	7.5
F91	4.448	15	10
Control	0	15	12.5

**Root wt. (mg) of 10 seedlings.

consisting of four subgroups: a) F14A; b) F36, F42, F55; c) F44, F91, F55A; d) F45. The second cluster consisting of four isolates with three subgroups: a) F22, F47; b) F82 ; c) F90.

Isolate F14A was found to be different from other isolates at molecular and virulence level. This purple coloured isolate was fast growing and avirulent and can be used in pathogen derived resistance (PDR) for management of seed discolouration of rice.

Seed borne *Fusarium* is a major yield constraint for long duration rice cultivars. The pathogen derived resistance (PDR) which has been evolved from cross protection is an effective and eco-friendly method for management of this pathogen. Virulence studies indicated that isolate F-14A is avirulent and molecular analysis lead to conclude that F-14A is genetically diverse from all other isolates. The vigour index of Utkalprabha seedlings, treated with *Fusarium* isolate

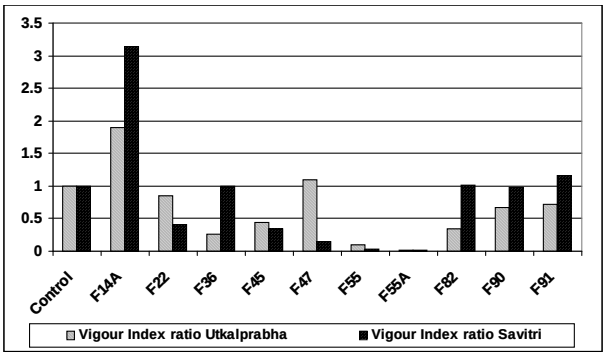

Fig. 1. Effect of *Fusarium* isolates on Vigour Index of rice cultivars

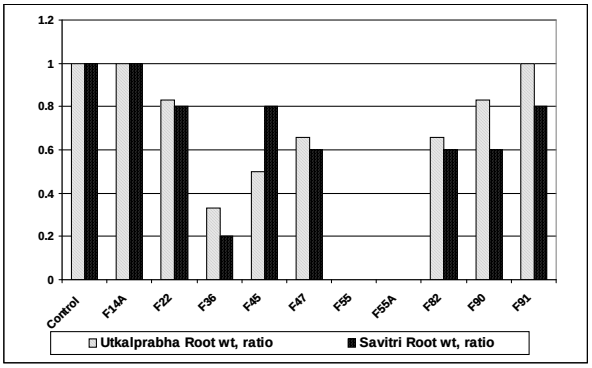

Fig.2. Effect of *Fusarium* isolates on root weight of rice cultivars

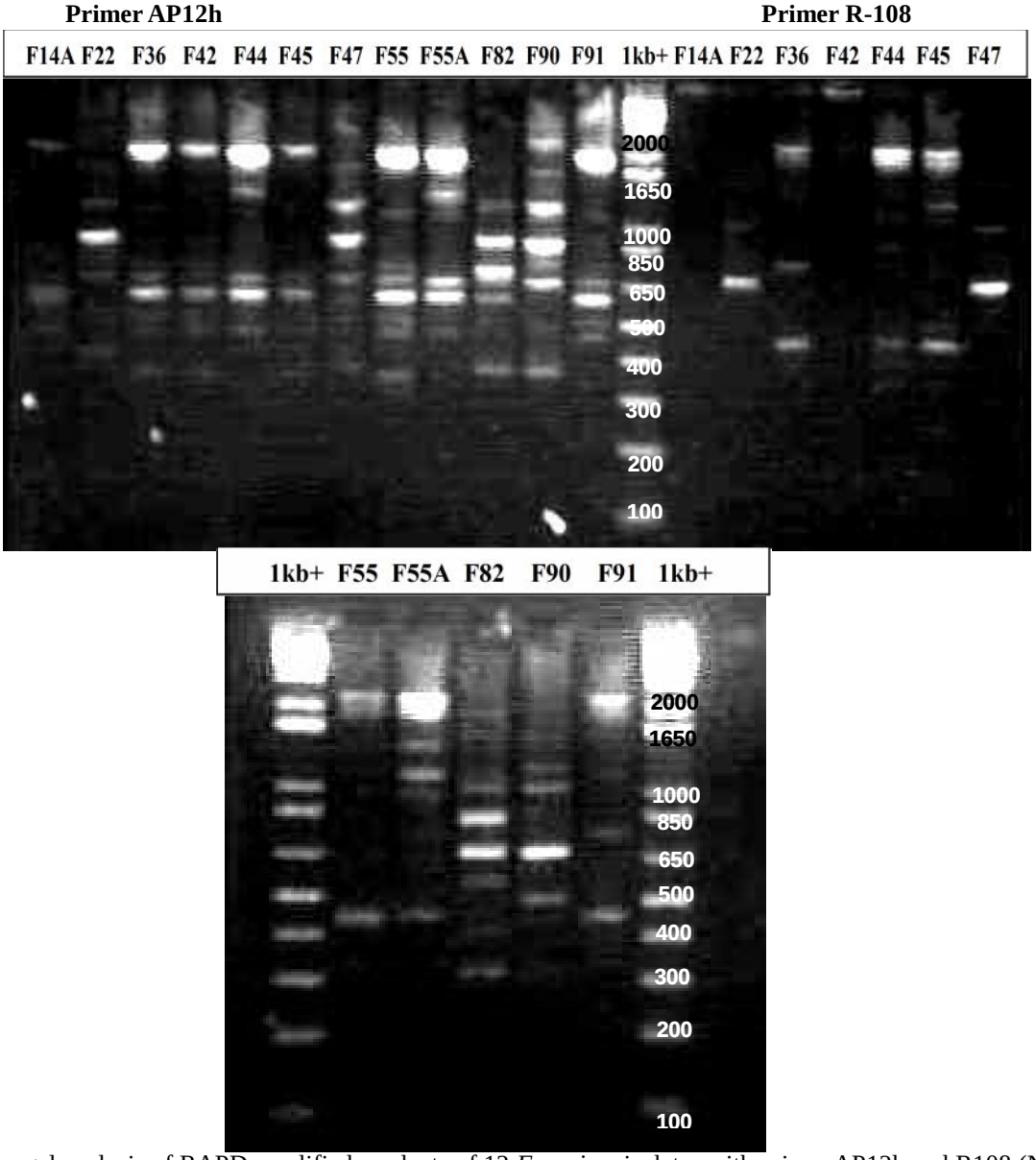

Fig. 3. Agarose gel analysis of RAPD amplified products of 12 *Fusarium* isolates with primer AP12h and R108 (Numbers correspond to isolates, 1kb+ molecular markers**)**

Table 3. Matrix for the similarity coefficients obtained between *Fusarium* isolates after the RAPD assessment.

	F14A	F22	F36	F42	F44	F45	F47	F55	F55A	F82	F90	F91
F14A	1											
F22	0.74	1										
F36	0.68	0.63	1									
F42	0.79	0.74	0.89	1								
F44	0.68	0.58	0.74	0.74	1							
F45	0.74	0.68	0.74	0.79	0.79	1						
F47	0.63	0.84	0.58	0.68	0.58	0.63	1					
F55	0.61	0.55	0.87	0.82	0.82	0.71	0.55	1				
F55A	0.55	0.5	0.71	0.66	0.82	0.66	0.5	0.84	1			
F82	0.55	0.76	0.55	0.66	0.55	0.61	0.76	0.53	0.42	1		
F90	0.55	0.71	0.39	0.5	0.5	0.61	0.76	0.42	0.37	0.68	1	
F91	0.66	0.61	0.87	0.76	0.82	0.76	0.55	0.79	0.79	0.58	0.47	1

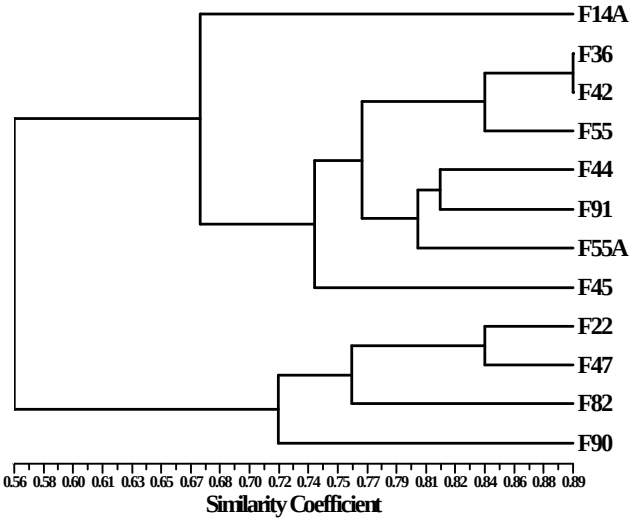

Fig. 4. Dendrogram derived from cluster analysis showing relationship among the 12 *Fusarium* isolates. Genetic similarity was obtained by RAPD markers.

number F-14A was almost double than the treated control. The vigour index of Savitri seedlings increased three times after treatment with F-14A. This suggests that isolate F-14A can be used for effective management of this disease by pathogen derived resistance.

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