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GENETIC VARIABILITY AND AGGRESSIVENESS IN *CURVULARIA LUNATA* ASSOCIATED WITH RICE-WHEAT CROPPING AREAS OF PAKISTAN

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Abstract

Curvularia lunata isolated from root and soil of both wheat and rice crops were identified and their aggressiveness was studied using aggressiveness analysis. Isolates of *Curvularia lunata* were genetically characterized using RAPD's. The investigations were based on two surveys of wheat and one survey of rice. In root aggressiveness analysis *Curvularia lunata* isolates were aggressive for rice than wheat. In foliar aggressiveness test the overall number of aggressive isolates was high on wheat. Random Amplified Polymorphism DNA (RAPD) was used to study the polymorphism and genetic variation within the population of fungi to establish correlation between aggressiveness, taxonomical and genetical characters of fungi. With RAPD analysis four groups were recognized and isolates were placed in different groups according to their banding pattern and aggressiveness behaviour. This study highlighted the correlation between aggressiveness, morphological and genetic variations of *Curvularia lunata.*

Introduction

Rice-wheat cropping system in Pakistan covers almost 2 million hectares and predominantly spreads across districts of Gujranwala, Sialkot, Norowal and Shiekhupura in the Punjab. The productivity of this system is reported to be in stagnation or to have declined in many areas, especially where a continuous rice-wheat rotation is followed. Biotic stresses that are an impediment appear to be very complex. Soil-borne pathogens, among others, are emerging as critical but are not well understood. These may limit nutrient uptake, internal water potential, photosynthesis and increase respiration, factors that are important for the productivity of a crop. A complex of soil-borne organisms particularly fungi cause diseases (Shamoun *et al.,* 1991; Schill *et al.,* 1994). Because of their wide host range and higher survival capacity, these are difficult to manage. These are not well understood in the rice-wheat system perspective. Some fungal pathogens are specific for rice or wheat. There are number of species that cause similar diseases in both rice and wheat. However, root rot in wheat and rice is caused by various fungi viz., *Fusarium* spp., *Rhizoctonia solani* or *Rhizoctonia oryzae, Bipolaris sorokiniana* or *Bipolaris sativum, Alternaria alternata, Helminthosporium* spp., *Curvularia* spp., and the oomycetes *Pythium* spp., and *Phytopthora infestans* (Blackie & Conroy 1994; Iftikhar *et al.,* 2003). This project was the start of a long-term study of fungal pathogens in rice-wheat cropping system in Pakistan. Therefore, one important aim was to establish a base line for later studies. Surveys of infection in the field were carried out. Fungal strains were isolated from soil, root and foliage. These strains were identified by classical methods and preserved as a culture collection. An important question was whether the same fungal strains infect on both rice and wheat. For this reason attention was focused

on fungal species that can infect both plants. Aggressiveness tests were carried out in the greenhouse on both rice and wheat varieties (Duveiller *et al.,* 2004). Classical methods often do not distinguish between isolates of the same species. Therefore DNA based methods should be applied to find out if the strains isolated from wheat and rice differ substantially from each other. The RAPDs method was chosen because of its simplicity and ability to provide taxonomical data. This study was concentrated on *Curvularia lunata* because it is common on both crops and its strains are particularly difficult to distinguish with classical methods. Research proposed in this study aims at achieving a better understanding of the causes of stagnating/declining yields in these systems and developing strategies to reduce losses caused by soil-borne fungi. Improved soil health resulting from control of fungi would contribute significantly to yield increase and income of the poorest farmers.

Materials and Methods

General protocol for wheat and rice sampling: Root samples of wheat and rice crops were collected from various fields (Table 1) and sample were taken at 10 points along a diagonal transect (Anon., 1996). At each sampling site, the samples were put in plastic and paper bags and transferred to the laboratory for further analysis. Assessment of root rot (0-3) and foliar blight (0-5) was done with the help of disease severity scales (Ledingham 1961; Anon., 1996).

Isolation, identification and preservation of fungi: Roots of rice and wheat were washed thoroughly in running tap water for 10-15 min., and cut into pieces, surface sterilized in 1% clorox for 1 min., rinsed three times in sterilized distilled water, dried on sterile blotting paper and plated on Potato Dextrose Agar (PDA) (Waskman & Fred 1992). The plates were incubated at 27°C for 3-4 days. Soil borne fungi were isolated from soil through soil dilution method at 10^{-3} dilution. The culture of root and soil fungi were purified and maintained on PDA slants at 27°C.

Aggressiveness analysis: After the isolation and identification of *Curvularia lunata*, isolates were evaluated for their aggressiveness and classified into different severity classes with the help of severity scales. The experiment was conducted under controlled conditions and the commercial varieties of wheat (Inqalab-91 and Chakwal–86) and rice (Basmati-386 and IRRI-6) were tested for the evaluation of aggressive isolates of root rot.

Agressiveness analysis of root rot: For the evaluation of root rot, the wheat and rice varieties nurseries were grown in sand for 6-8 days. Single spore cultures of *Curvularia lunata* were grown on potato dextrose agar (PDA) for 6-10 days. Conidial suspension of *Curvularia lunata* was counted with a hemocytometer and diluted with autoclaved distilled water. One drop of tween 20 was added. After 6-8 days of the sowing of wheat and rice seeds, plant roots were removed from sand very carefully and inoculated with spore suspension and again transplanted in small pots. The plants were planted in small pots and in pots autoclaved soil mix consisting of silt loam, sand and meat moss (1:1:1 by volume). In aggressiveness experiment for wheat plants the temperature was 20°C to 22°C and for rice plants 30 to 32°C. After four weeks, plants were uprooted and roots were washed with water and browning and blackening on roots were observed with the help of severity scale 0-3 (Ledingham, 1961).

Table 1. Origin of *Curvularia lunata* isolates, which were isolated from the root and the soil of rice and wheat crops at the different stages of plant growth.

Fungi	Number	Location	Stage	Plant Parts	Crop
Curvularia lunata	C_1	Muridke	Heading	Root	Rice
Curvularia lunata	C_2	MONA	Heading	Root	Rice
Curvularia lunata	C_3	Gujranwala	Heading	Root	Rice
Curvularia lunata	C_4	MONA	Heading	Root	Rice
Curvularia lunata	C_5	MONA	Heading	Root	Rice
Curvularia lunata	C_6	Sheikhupura	Heading	Soil	Rice
Curvularia lunata	C_7	Muridke	Heading	Root	Rice
Curvularia lunata	C_8	MONA	Heading	Root	Rice
Curvularia lunata	C_9	Muridke	Heading	Root	Rice
Curvularia lunata	C_{10}	Narowal	Heading	Root	Rice
Curvularia lunata	C_{11}	Gujranwala	Heading	Root	Rice
Curvularia lunata	C_{12}	Muridke	Heading	Root	Rice
Curvularia lunata	C_{13}	Muridke	Booting	Root	Wheat
Curvularia lunata	C_{14}	Sheikhupura	Heading	Root	Rice
Curvularia lunata	C_{15}	Islamabad	Heading	Root	Rice
Curvularia lunata	C_{16}	Sialkot	Booting	Root	Wheat
Curvularia lunata	C_{17}	Sialkot	Booting	Root	Wheat
Curvularia lunata	C_{18}	Sialkot	Booting	Root	Wheat

Aggressiveness analysis of foliar blight: Isolates of *Curvularia lunata* were also tested for their foliar aggressiveness. Inocula were produced by the method used by Lamari & Bernier (1989). Conidia plus mycelial suspension were prepared by flooding the plates with sterile distilled water and scraping the colony with a glass slide. A drop of tween 20 was added per 100 ml of suspension, and the concentration was determined with a hemocytometer. Eight seeds of wheat and rice varieties were planted in plastic pots containing sterilized soil in a green house. Seedlings were thinned after emergence to five plants per pot and then transferred to controlled-experimental conditions. Seedlings of rice and wheat at the two-leaf stage were sprayed with conidial suspension. Control plants were sprayed with distilled water. Rice and wheat plants were covered with plastic bags for high humidity and bags were removed after 30 hours. Disease severity was estimated on inoculated leaves 7 day after inoculation by 0-5 severity scale. The pathogen was re-isolated by the method described above in pathogenicity.

Aggressiveness data analysis: All isolates of fungi were selected as weak and highly aggressive isolates on the basis of pathogenic reaction. Data were analyzed using the SAS computer software package (Statistical Analysis System Institute Inc., Cary, NC). Disease severity means of all isolates were subjected to an Analysis of Variance using the SAS ANOVA procedure. Cluster analysis was done by SAS CLUSTER procedure, using the centroid method. In centroid method the distance between two clusters is defined as the squared Euclidian distance between their centroids or means. The variance among wheat and rice varieties in disease reaction to soil-borne fungi was regressed against mean aggressiveness of the isolates. Similarly, the variance among isolates in aggressiveness to wheat and rice varieties was regressed against mean resistance of the varieties. This method was proposed by Carson (1987) to predict modes of host-pathogen interaction in the pathosystem of soil-borne fungi.

Genetic characterization of fungi: Each isolate of fungi was grown on potato broth. Mycelium was harvested by filtration on Whatmann No 1 filter paper and frozen at -20°C for few minutes. The frozen mycelia were grounded in liquid nitrogen (Rogers &Bendich, 1985). Phenol Chloroform and isoamyl-alcohal extraction was carried out for the extraction of DNA.

DNA amplification: For PCR amplification, five 10-mer random primers were selected viz., P1 (5´-AGGAGGACCC-3´), P2 (5´-ACGAGGGACT-3´), P14 (5´-CCACAGCACG-3´), PE7 (5´-AGATGCAGCC-3´) and PE20 (5´-AACGGTGACC-3´), (Altomare *et al.*, 1997). The amplification was performed in a thermal cycler program:Cycle-1: 94°C for 10 minute, Cycle-2 : 97°C for 15 minute, 36°C for 1 minute, 72° C for 2 minute Repeat for 40 times, Cycle-3: 72°C for 10 minute and Cycle-4: 4°C for 30 minute.

Data analysis of amplified products: PCR products were analyzed by gel electrophoresis on 1.4 % agarose and detected by ethidium bromide. After washing of gel, the photograph was taken with UV transilluminator. DNA bands on gels were scored as present (1) or absent (0) for all isolates and species studied. The 0/1-matrics were analyzed with 'PHYLIP' phylogeny inference package version 3.57c.

Results

Aggressiveness of *Curvularia lunata* for root rot

Wheat: Analysis of Variance (ANOVA) of aggressive behavior of 18 isolates of *Curvularia lunata* was analyzed on the two commercial varieties (Inqalab-91 & Chakwal-86) of wheat showed non-significant effects of varieties, replications and varieties x replication. There was highly significant effect of isolates, replications x isolates and varieties x Isolates. The aggressive behaviour of isolates was further investigated by cluster analysis. Two main groups A and B were identified (Fig. 1). Majority of the isolates were non-aggressive and fall in group A; these included C1, C3, C4, C7, C8, C10, C13 and C16. Isolates C2, C5, C6, C11 and C18 were highly aggressive on both varieties. Isolates C9, C12, C14, C15 and C17 were slightly aggressive on both varieties of wheat crop; however, within group they also showed different behavior.

Rice: Analysis of Variance (ANOVA) of *Curvularia lunata* isolates on the both varieties of rice showed that there was non-significant effect of varieties, replications, varieties x replications, varieties x Isolates and replications x Isolates. There was highly significant effect of Isolates. Two groups A and B were identified by cluster analysis of two varieties of rice using the centroid method (Fig. 2). Figure shows that in group A all the isolates were non aggressive on the both varieties of rice crop. Group B has further subgroups (B1 and 2).

Comparison of *Curvularia lunata* for root rot on rice and wheat: The scatter diagram is showing the aggressiveness of isolates on wheat and rice crops at difference level of aggressiveness (Fig. 3). Overall, the number of aggressive isolates was high in rice than wheat.

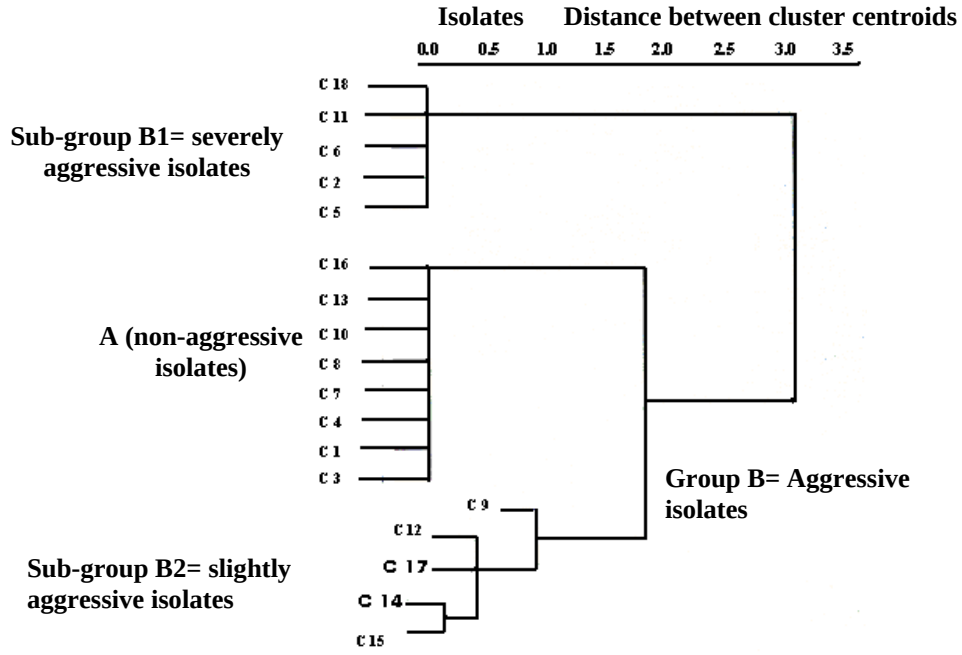

Fig. 1. Dendrogram showing similarity and successive clustering of isolates of *Curvularia lunata* based on their aggressiveness on two wheat varieties.

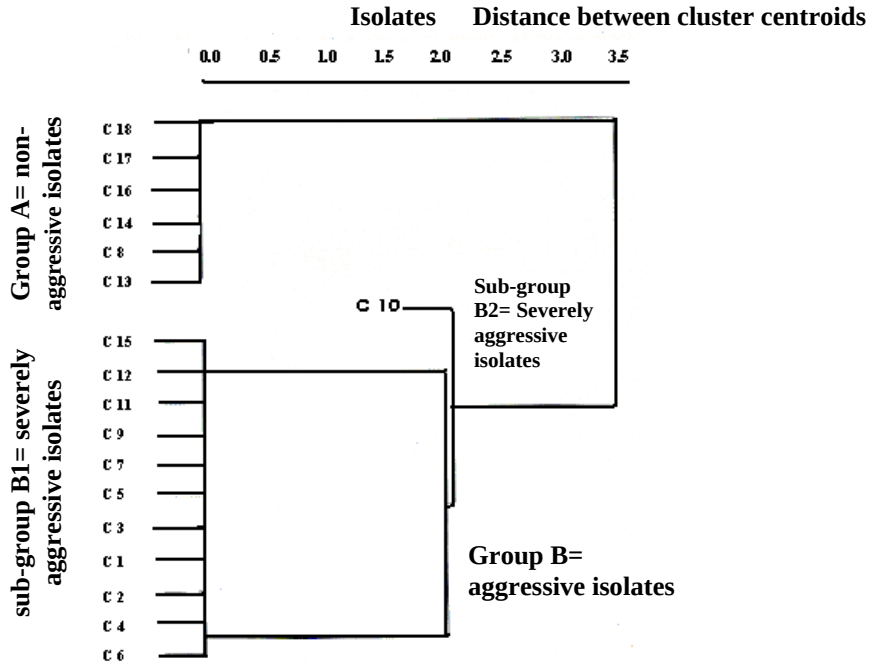

Fig. 2. Dendrogram showing similarity and successive clustering of isolates of *Curvularia lunata* based on their aggressiveness on two rice varieties.

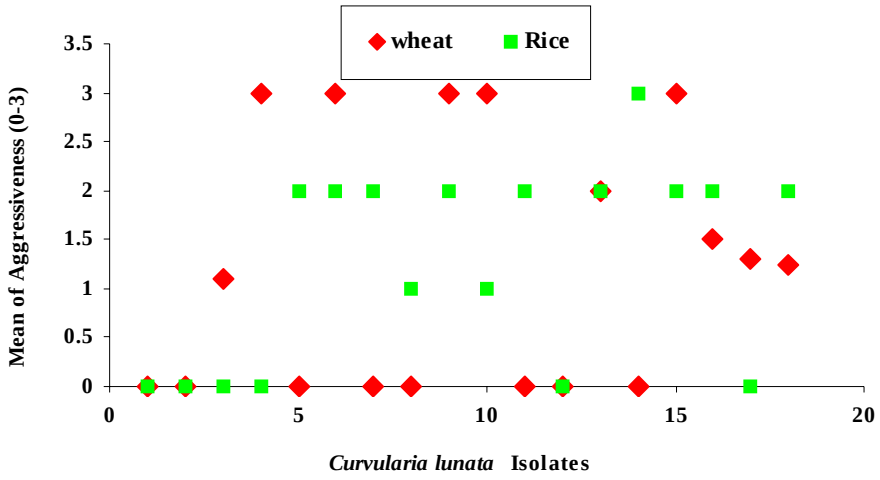

Fig. 3. Mean aggressiveness of *Curvularia lunata* isolates for root rot on wheat and rice varieties.

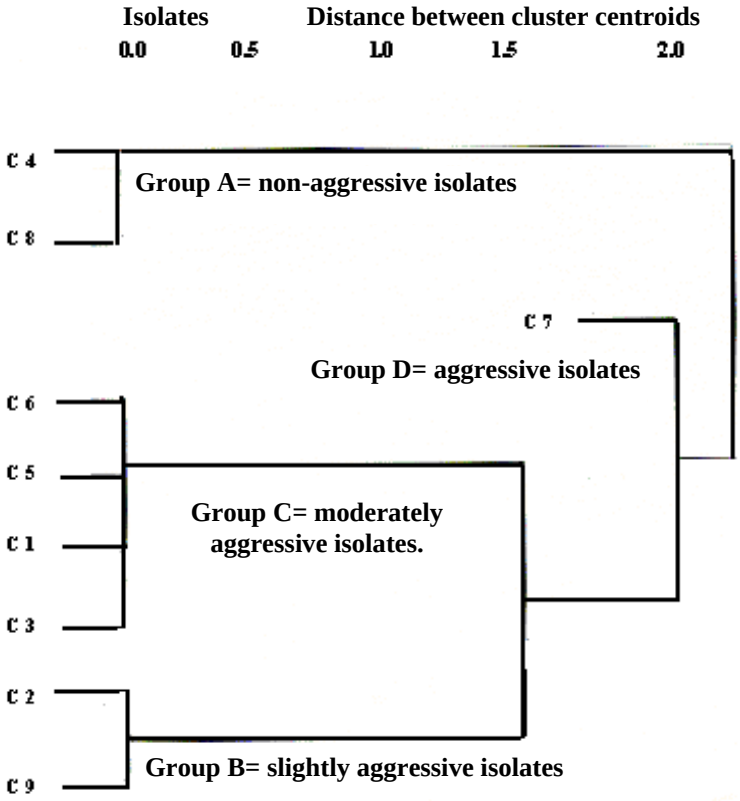

Fig. 4. Dendrogram showing similarity and successive clustering of isolates of *Curvularia lunata* based on their aggressiveness on two wheat varieties.

Rice: The aggressiveness behavior of 9 isolates of *Curvularia lunata* was analyzed by Analysis of Variance on two commercial varieties of rice crop viz., Basmati-385 and IRRI-6. There was no significant effect of replications, varieties x replications and replications x Isolates interaction. There was a highly significant effect of varieties, Isolates and varieties x isolates interaction. Four groups A, B, C and D of similar isolates were identified by clustering analysis of the combined experiments using the centroid method. In group A isolates were non-aggressive. In group B isolates were slightly aggressive. In group C isolates were moderately aggressive and group D aggressive (Fig. 5).

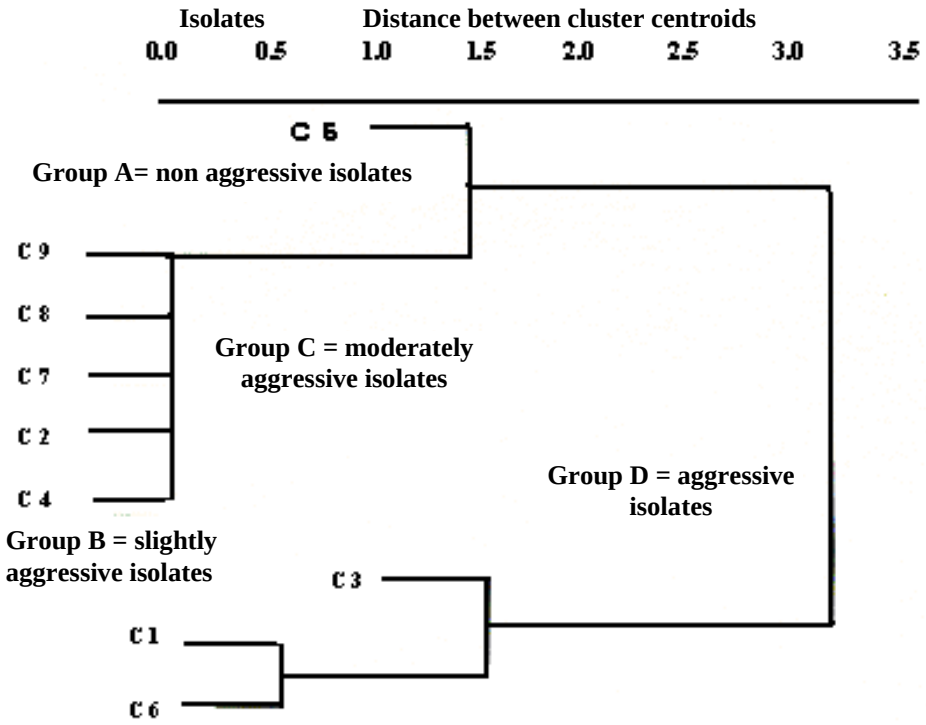

Fig. 5. Dendrogram showing similarity and successive clustering of isolates of *Curvularia lunata* based on their aggressiveness on two rice varieties.

Comparison of *Curvularia lunata* on rice and wheat: Overall, more number of isolates showed aggressiveness on wheat than rice (Fig. 6). Only one isolate showed same level of aggressiveness on both wheat and rice. The level of aggressiveness was also high in wheat as compared to rice.

RAPD study of *Curvularia lunata*: DNA was extracted from the total of 12 *Curvularia lunata* isolates and RAPD pattern with five primers was studied. Depending on the DNA template-primer combination. DNA fragments were amplified, between the range of 200bp to 2500bp. Major bands were amplified with high reproducibility. Same level of variability in the banding pattern was found between the isolates of *Curvularia lunata*. With all primer data 59 bands were produced. On analysis phylogenetic dendrogram produced and showed the grouping of non-aggressive isolates. Hence, the correlation has shown between the

isolates of aggressiveness and non aggressiveness genetic data. In this experiment all primers produced both inter and intra group polymorphism, indicating high level of genomic variability within the isolates of *Curvularia lunata* based on visual examination of patterns and the analysis of molecular variance. Within group, there was apparent relationship between aggressiveness and the clustering pattern. All the isolates of *Curvularia lunata* were similar in culture morphology. Figure 7 shows that phylogenetic dendogram based on the single linkage method evidence of four phonetic groups (1, 2, 3, 4). The isolate C4, C5, C9, C11, C12 in cluster 1. In cluster 2 the isolates C8, C13 and C16 were non aggressive on wheat and rice varieties. In cluster 3 only C14 and it was non aggressive on rice varities. The cluster 4 is divided in sub-cluster 4i and 4ii and isolates C3, C7 and C11 were non aggressive on wheat varieties. The amplification profile of five primers clearly differentiated isolate from each other in banding pattern. Similarity between isolates and the number of unique genotypes were found primer dependent.

Discussion

From wheat and rice roots 4 and 14 isolates were isolated respectively. On wheat 10 isolates and on rice 12 isolates were aggressive. On wheat 8 and on rice 6 isolates were non-aggressive. On wheat and rice varieties 3 isolates of *Curvularia lunata* showed the same non-aggressive behaviour (Table 2).

From foliage of wheat no *Curvularia lunata* isolate was isolated and from rice 9 isolates were isolated. On wheat varieties the aggressive behaviour of *Curvularia lunata* isolates was more than rice. On wheat and rice varieties, 2 isolates showed the same non-aggressive behaviour on both varieties of crops (Table 3).

During this study, it was observed the morphological differences between *Curvularia lunata* foliar and root isolates were less. They have same conidia size, shape, colour and morphology. In aggressiveness tests *Curvularia lunata* isolates showed aggressiveness on both rice and wheat varieties. Possible factors that contributed important role in aggressiveness were environment, inoculum and temperature condition (Hodges, 1972).

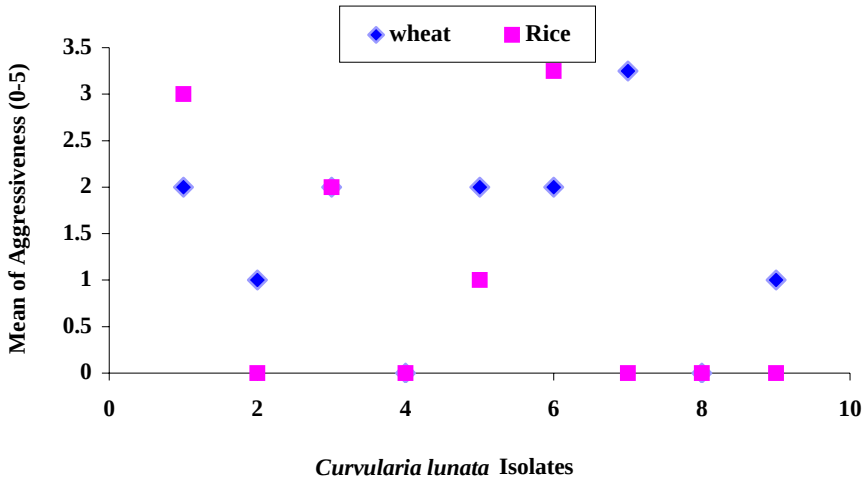

Fig. 6. Mean aggressiveness of *Curvularia lunata* isolates for foliar blight on wheat and rice varieties.

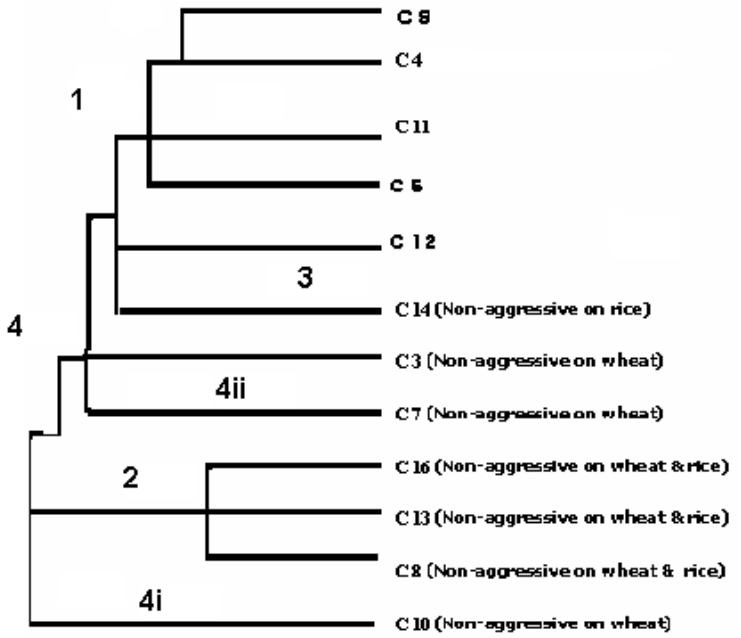

Fig. 7. Phylogenetic dendogram of the isolates of *Curvularia lunata* based on RAPD fingerprintinting of Primer P1,P2,PE7, P14 and PE20.

Table 2. The aggressivene behavior of *Curvularia lunata* on roots of rice and wheat varieties.

Re-isolation of *Curvularia lunata*	**Number**
From wheat	4
From rice	14
Aggressive isolates	
On wheat	10
On rice	12
Non-aggressive isolates	
On wheat	8
On rice	6
Non-aggressive on both crops	**3**

Table 3. The aggressive behavior of *Curvularia lunata* on foliage of rice and wheat varieties.

Re-isolation of *Curvularia lunata*	**Number**
From wheat	0
From rice	9
Aggressive isolates	
On wheat	7
On rice	4
Non-aggressive isolates	
On wheat	2
On rice	5
Non-aggressive on both crops	**2**

During this study use of five randomly selected primers evaluate the genetic diversity among the collection of *Curvularia lunata* isolates sampled from the different locations of rice and wheat. The RAPD analysis showed a high level of genetic variations among the isolates of *Curvularia lunata* though they had been isolated from two different crops which are existing in summer or winter season (Berretta *et al.,* 1998; Castrillo & Brooks, 1998). The primers used by Berretta *et al.,* 1998; Castrillo & Brooks, 1998 in the RAPD analysis are different from those used in the present study. With the primer used in present study showed the high genetic variations. The genomic regions amplified with these five primers we used could represent conserved regions, while the highly variable regions could have been amplified with the primers used by Berretta *et al.,*1989; Castrillo & Brooks, 1998. Several amplified DNA fragments were similar with population of *Curvularia lunata*, whether they had been isolated from rice and wheat crops roots and soil. With RAPD analysis all the isolates of *Curvularia lunata* confirmed the level of similarities and difference and also identified in grouping. In all primer data analysis showed a good relationship between the morphological, aggressiveness and genetical characters. All non-aggressive isolates are present in very close to each other in cluster and the analysis is showing a good relationship as shown by other workers (Shamoun & Ekramoddoullah, 1991; Meijer *et al.,* 1994). The RAPD fragments observed in our analysis, probably being conserved regions, could serve as informative probes in RFLP analysis. Six of the seven RAPD products of *Fusarium solani* did turn out to represent unique sequences and were useful in identifying markers for different mating groups (Crowhurst *et al.,* 1995).

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