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# Analysis of Variation in *Alternaria alternata* by Pathogenicity and RAPD Study

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Abstract

*Alternaria alternata* were isolated and identified from root, foliage and soil of both wheat and rice crops and their aggressiveness was studied using aggressiveness analysis. Isolates of *Alternaria alternata* were genetically characterized using RAPD's. The investigations were based on surveys of wheat and rice crops in the rice-wheat cropping areas of Pakistan. The study showed that *Alternaria alternata* is root rot causing fungi and during root rot aggressiveness analysis the isolates showed higher aggressiveness on rice varieties than on wheat but in foliar aggressiveness the overall number of aggressive isolates was higher on wheat varieties than on rice. In genetic characterization Random Amplified Polymorphism DNA (RAPD) was used to study the polymorphism and genetic variation within the population of *Alternaria alternata* that established correlation between aggressiveness and genetical characters of fungi. *Alternaria alternata* tree is constructed based on the pattern of bands. This study highlighted the correlation between morphological, aggressiveness and genetic variations of *Alternaria alternata.*

Key words: *Alternaria alternata*, host-pathogen relations, foliar blight, root rot, RAPDs-PCR

## Introduction

Rice-wheat cropping system in Pakistan covers almost 2 million hectares and pre-dominantly spreads across districts of Gujranwala, Sialkot, Norowal and Shiekhupura in 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 (Blackie and Conroy, 1994; Schill *et al.*, 1994). These may limit nutrient uptake, internal water potential, photosynthesis and increase respiration and factors that are important for the productivity of a crop. A complex of soil-borne organisms, particularly fungi, cause diseases. Because of their wide host range and higher survival capacity, they are difficult to manage. These are not well understood in the rice-wheat system perspective. Some fungal pathogens are specific for rice or wheat. Yield stagnation or decline is a problem in rice-wheat systems in many areas, reducing rural income and food security (Kumar *et al.*, 1999; Garni *et al*., 2001 and Bhandari *et al.*, 2002). The basic objective of this project was to achieve a better understanding of the soil-borne fungal pathogen and associated diseases in rice-wheat cropping system. During this study first of all three main surveys were conducted in rice wheat cropping area of Punjab and diseases were also recorded with primary emphasis on root rot and foliar blight. Broad spectrum of soil-borne pathogenic and non-pathogenic fungi were isolated in laboratory with the help of PDA and blotter method. The main purpose of this study was to understand the biology of soil-borne fungal pathogens which occur as complexes in rice-wheat cropping system. In Pakistan very little research has been conducted before this study and the nature of soil-borne fungi in rice-wheat cropping system is poorly understood and little quantified. Improvement of soil health can control pathogens and significantly contribute the yield and income of the poorest farmers. The soil-borne fungi survive as chlamydospores in the soil or in infested plant debris. They are good saprophytes in tissues which they previously parasitized, and can multiply rapidly on infested cereal and grass residues. Chlamydospores

formed in conidia or mycelium may persist in soil for months. These germinate and produce hyphae, which infect mainly through crown roots and through wounds sustained during crown root emergence. The pathogen then invades internodal tissues. Moisture is essential for infection, but moisture stress during the late boot phase and heading enhances the disease, hence the name root rot. Crop rotation to nonhost crops and avoiding rice-wheat, help limit the buildup of pathogen populations in the soil (Ali *et al.*, 1998). Many of the fungi isolated from plant suffering from root rot or foliar blight were primarily soil-borne but they are also known to be seed transmitted pathogens. The most accessible means of controlling such disease is through selection of healthy seeds for planting because during survey one can get information on the source of seed. This is the first report about the soil-borne diseases of wheat and rice crop in rice-wheat cropping system perspective. The commonality of occurrence and aggressiveness of pathogenic fungi both on rice and wheat clearly show a strong need for diversification of the rice-wheat system to break the perpetuation of these pathogens in the system (RWC-CIMMYT, 2003). Setting up a genetic fingerprint for the soil-borne fungi will also enable to identify the range of genetic variation within the species by providing a key to which other fungi strains and populations from other locations can be compared (Tcherneva *et al*., 2000). Molecular techniques based on DNA analysis seem to offer a wide range of advantages (Zeise and Tiedemann, 2002). The RAPD technique will allow to develop specific probes to study biodiversity not only at the level of species but also at the level of individual populations. The RAPD technique is easier and faster than other methods but may have problems with the reproducibility between laboratories. In this work, optimal conditions like precise extraction and constant amplification allowed to obtain reproducible results. The generation of DNA fingerprints using the randomly amplified polymorphic DNA techniques (RAPD) is particularly useful because no prior genetic knowledge of the target organism is required (Delye *et al.*, 1997). This investigation has provided a very useful tool in the form of RAPD markers to understand and to distinguish fungal species, races, pathotypes and strains of multitude of pathogens affecting rice-wheat system.

## Experimental

### Materials and Methods

**General protocol of wheat and rice sampling.** Foliar and root samples of wheat and rice crops were collected from various fields and sample were taken at 10 points along a diagonal transect (IRRI, 1996). At each sampling site, carefully taken 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.

**Isolation, identification and preservation of fungi.** Foliar samples that possessed disease symptoms were cut into small pieces. The identity of the sample was recorded on the dry filter paper previously placed in the bottom of each Petri plate. Autoclaved sterilized distilled water was added to moisten the filter paper. Leaf sections were surface sterilized by dipping them in 3% Clorox for 1 min and then three times rinsed with autoclave distilled water. Plates were placed at 25°C for 24 hours under photoperiod and then at 18°C for 24 hours in dark period. After continuous light and dark period, the presence of fungi on leaf sections was recorded under stereomicroscope (De Wolf *et al.*, 1998). Roots were separated and washed thoroughly in running tap water for 10–15 min and then 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) (Usmani and Ghaffar, 1982). The plates were incubated at 27°C for 3–4 days. The cultures of root fungi were purified and maintained on PDA slants at 27°C. Soil borne fungi were isolated from soil through soil dilution method (Waskman and Fred, 1992) at $10^{-3}$ dilution. The culture of fungi were purified and maintained on PDA slants at 27°C.

**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 and Bendich, 1985). Phenol-chloroform and isoamyl alcohol extraction was carried out for the extraction of DNA.

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

**Analysis of amplified products.** PCR products were analyzed by gel electrophoresis on 1.4% agarose and stained 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

There are number of fungi that cause similar diseases in both rice and wheat. However, root rot in wheat and rice is caused by *Alteraria alternata*. 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 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 rice and wheat varieties. Classical methods often do not distinguish between isolates of the same species. Therefore DNA based methods was applied to find 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 differentiate the isolates. This work was concentrated on *Alternaria alternata* because they are common on both crops and 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 plant fungi. Improved soil health resulting from control of fungi would contribute significantly to yield increase and therefore the income of the poorest farmers.

**Aggressiveness of *Alternaria alternata* for root rot on wheat and rice.** The aggressive behavior of 17 isolates of *Alternaria alternata* was analyzed by Analysis of Variance (ANOVA). There was no significant effect of varieties, replications, isolates, varieties x replications and replication x isolates. There was significant effect of varieties x isolates. Two main groups (A and B) of similar isolates of *Alternaria alternata* were identified by cluster analysis. Group B has further sub-groups (B1, B2 and B3). The dendrogram shows the behavior of isolates on both varieties of wheat. In group A all the isolates are non-aggressive and has more number of isolates. In group B all the isolates are from slightly aggressive to severely aggressive (Fig. 1). Isolate A2, A3, A7, A9, A10, A11 and A13 were non aggressive on both varieties. Isolates A1 and A6 were slightly aggressive, isolates A4, A5, A15 and A16 moderately aggressive, and isolates A8 and A14 were severely aggressive on both varieties of wheat. Isolates A12 and A17 showed more aggressive behavior on Chakwal-86 as compared to Inqalab-91.

In rice the Analysis of Variance (ANOVA) for aggressiveness showed that there was no significant effect of varieties, replications, varieties x replications and replications x isolates. There was a very highly significant effect of isolates and varieties x isolates. Two groups (A and B) were also identified by cluster analysis of the combined experiment using the centroid method. Group A containes all isolates non-aggressive on both varieties of rice. Isolates A12, A14, A15, A16 and A17 were non aggressive on both varieties of rice. Group B is further divided in sub-groups B1, B2 and B3. In groups and sub-groups the isolates are in different aggressive classes (Fig. 2). Isolates A3 and A10 were slightly aggressive on both varieties of rice. Isolates A1 and A6 were severely aggressive on both varieties of rice. Comparison of *Alternaria alternata* on rice and wheat demonstrated that the total number of aggressive isolates was higher in rice than wheat. Similarly, moderately aggressive isolates were more often in case of rice than wheat.

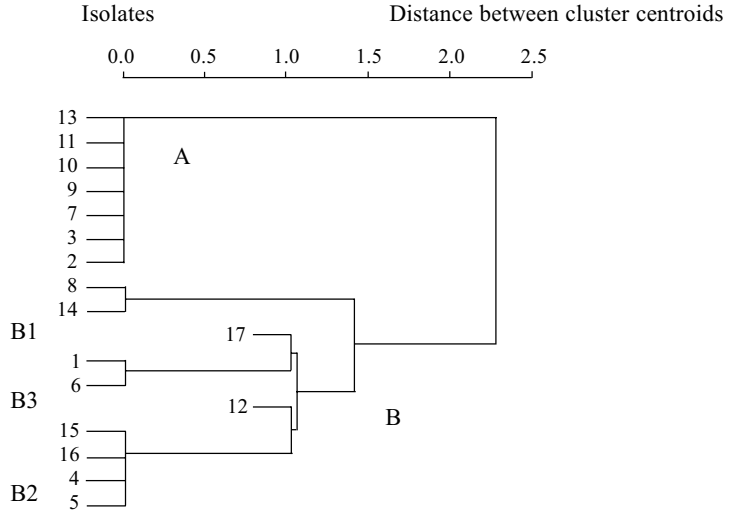


Fig. 1. Dendrogram showing similarity and successive clustering of *Alternaria alternata* isolates based on their aggressiveness on two wheat varieties.

Group A = non-aggressive isolates, group B = aggressive isolates, sub group – B1 = severely aggressive, sub-group B2 = moderately aggressive isolates and sub-group B3 = slightly aggressive isolates.

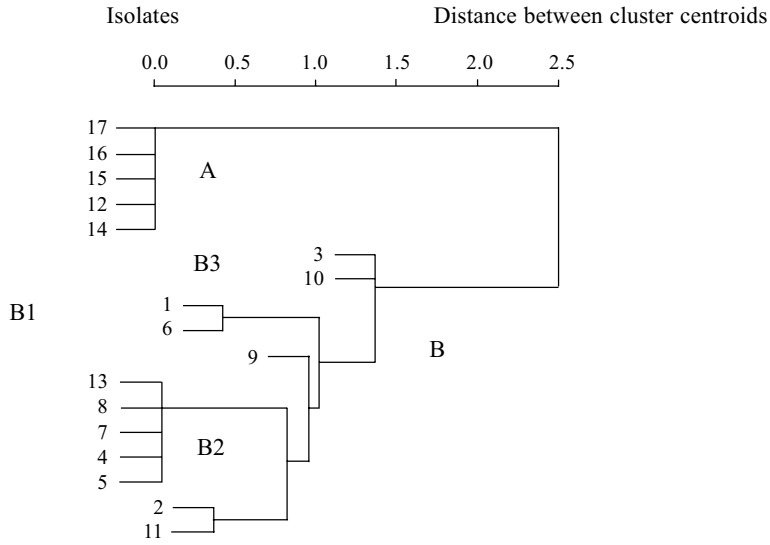


Fig. 2. Dendrogram showing similarity and successive clustering of *Alternaria alternata* isolates based on their aggressiveness on two rice varieties.
Group A = non-aggressive isolates, group B = aggressive isolates, sub-group B1 = severely aggressive isolates, sub-group B3 = slightly aggressive isolates, sub-group B2 = moderately aggressive isolates.

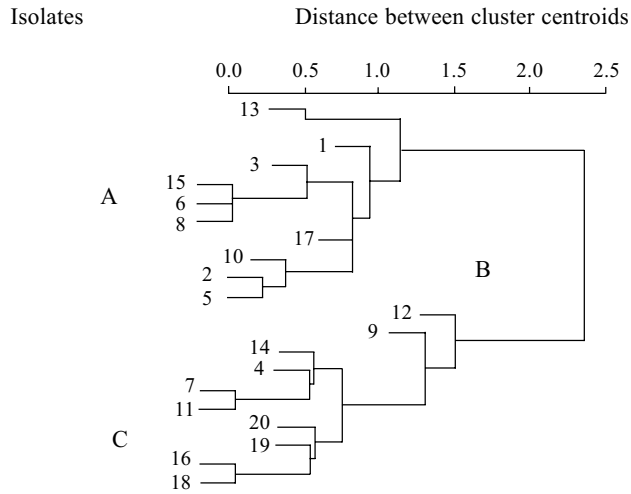


Fig. 3. Dendrogram showing similarity and successive clustering of *Alternaria alternata* isolates based on their aggressiveness on two wheat varieties.
Group A = slightly aggressive isolates, group B = moderately aggressive isolates, group C = aggressive isolates.

**Aggressiveness of *Alternaria alternata* for foliar blight on wheat and rice.** All isolates showed aggressiveness to both varieties of wheat while in rice some isolates were non-aggressive. The aggressive behavior of 20 isolates of *Alternaria alternata* was analyzed by Analysis of Variance (ANOVA) on two commercial varieties of wheat crop namely Inqalab-91 and Chakwal-86. 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. Three groups A, B and C of similar isolates were identified by clustering analysis of the combined experiments using the centroid method. In group A isolates were slightly aggressive. In group B isolates were moderately aggressive and isolates in group C were aggressive (Fig. 3).

In rice the aggressive behavior of 20 isolates of *Alternaria alternata* was analyzed by Analysis of Variance (ANOVA) on the two commercial varieties of rice crop namely 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. Three main groups A, B

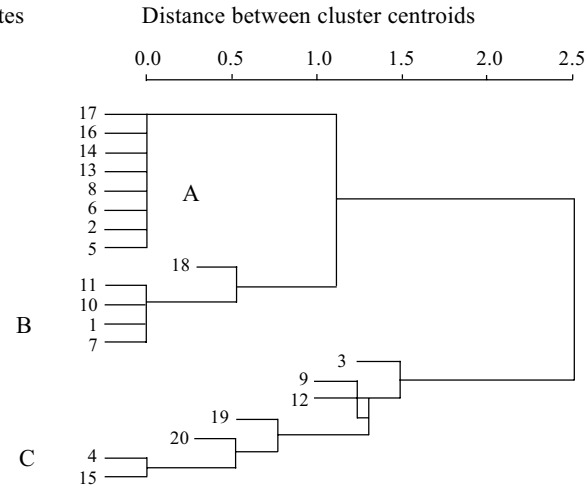


Fig. 4. Dendrogram showing similarity and successive clustering of *Alternaria alternata* isolates based on their aggressiveness on two rice varieties.
Group A = non-aggressive isolates, group B = slightly aggressive isolates, group C = moderately aggressive isolates.

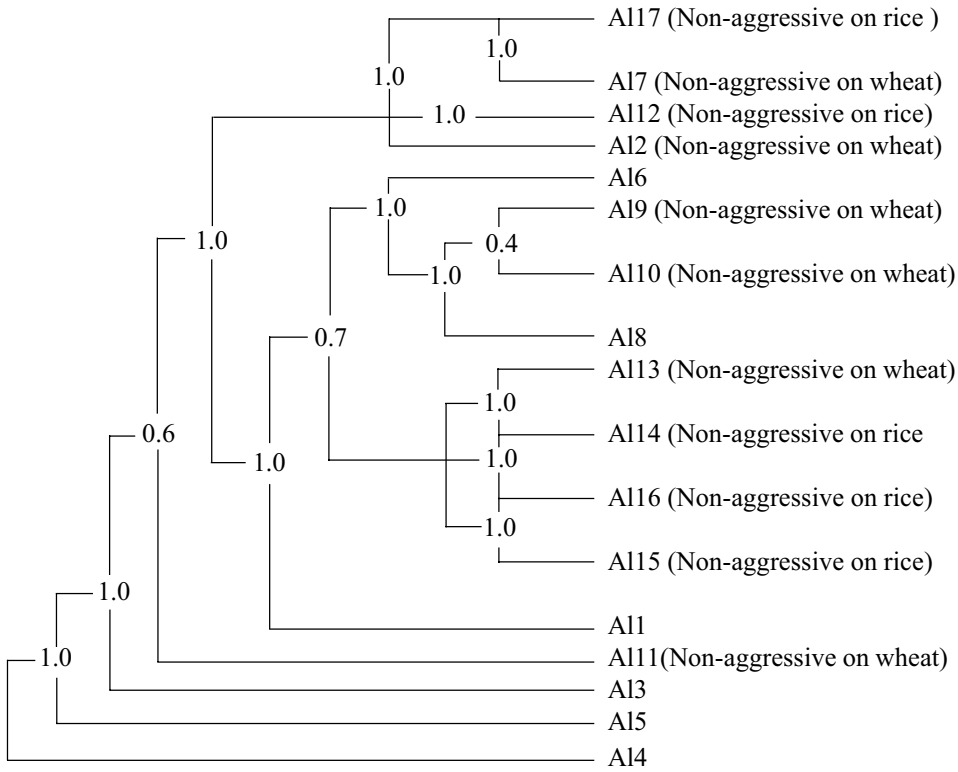


Fig. 5. Phylogenetic dendrogram of *Alternaria alternata* isolates based on RAPD fingerprinting of Primer P1, P2, PE7, P14 and PE20

and C of similar isolates were identified by clustering analysis of the combined experiments using the centroid method. In group A isolates were non-aggressive and in group B isolates were slightly aggressive and in group C isolates were moderately aggressive (Fig. 4). A comparison of aggressiveness of *Alternaria alternata* on rice and wheat foliage is showed in scatter diagram in Fig. 5. The level of aggressiveness was high on wheat as compared to rice. There were only two equi-aggressive isolates on rice and wheat.

**RAPD study of *Alternaria alternata*.** Five primers which sequences were given in Materials and methods were used for the study of genetic variations in 17 isolates of *Alternaria alternata* and to obtain the amplified products for numerical analysis. From *Alternaria alternata* DNA was extracted with CTAB

method which was found the best method for the extraction of DNA from *Alternaria alternata* strains. The all amplified bands were ranged in size from 400 bp to 2500 bp that represent a correlation produced between morphological, aggressiveness and banding patterns in species of *Alternaria alternata*. The Fig. 5 shows a clusters and different banding pattern in all the *Alternaria* species. Most of the cultures of *Alternaria alternata* are similar in shape of genetic characters and reflect aggressiveness.

## Discussion

*Alternaria alternata* is a soil-borne pathogen which causes root rot and foliar blight. During surveys it was isolated from roots of both wheat and rice samples. In total, 9 isolates were isolated from wheat and 8 isolates from rice. Overall the level of aggressiveness of *Alternaria alternata* isolates on the roots of wheat and rice showed that aggressiveness was high on rice as compared to wheat. 7 isolates were non-aggressive for wheat and 5 isolates were non-aggressive for rice. There were no isolates of *Alternaria alternata* that showed the same non-aggressive behaviour on rice and wheat varieties. During survey from wheat foliage, 19 isolates were isolated and from rice only one was isolated. In foliar aggressiveness test all isolates were aggressive on wheat but on rice only 12 isolates were aggressive. No isolates showed the same non-aggressive behaviours on both crops (wheat and rice) varieties. All isolates of *Alternaria alternata* root rot and foliar blight were quite varied in their origin and represent a wide range of aggressiveness phenotype. We showed that aggressiveness analysis was useful in understanding the wheat and rice varieties and *Alternaria alternata* in reference to host-pathogen relationship and also proved to be useful in determining the similarity of wheat and rice varieties, based on their reactions to *Alternaria alternata* (Lebeda and Jendrulek, 1987). Five RAPDs primer were used for the study of genetic variability of *Alternaria alternata.* During this study it was not possible to get good amplification products with one primer because only few DNA bands were amplified in some strains of *Alternaria alternata*. It was also observed with all primer that made different numbers of amplifications products were and different phylogenetic tree was produced. Phylogenetic tree obtained with all primers was entirely different than this obtained with one primer. In all primer phylogenic tree different clusters were produced and it showed that all non-aggressive isolates are present in one cluster on rice but one isolate was present that was non-aggressive on wheat. In other cluster two isolates that were non-aggressive on wheat were present. Some isolates, Al17, Al7, Al12 and Al2, non aggressive on wheat and rice were present in the same cluster. Results showed there that was no single marker that could differentiate isolates of *Alternaria* with a high fidelity, RAPD were generally useful in establishing a relationship between isolate and host (Crowhurst *et al.*, 1991). Similarly Petrunak *et al.* (1992) detected a polymorphism in *Alternaria solani* and *Alternaria alternata* isolates and used one primer P248 for RAPD study and generated DNA polymorphisms that distinguished the *Alternaria* spp. Aggressiveness analysis of rice and wheat crops proved that some isolates of *Alternaria alternata* showed the same aggressiveness behaviour on the varieties of both crops but when genetic analysis was done by RAPDs then again some isolates showed a good correlation among DNA banding pattern and aggressiveness.

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