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BIOLOGICAL PATHOTYPING AND RAPD ANALYSIS OF *ASCOCHYTA RABIEI*, FROM VARIOUS CHICKPEA GROWING AREAS OF PAKISTAN

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ABSTRACT

Blight disease caused by the fungus *Ascochyta rabiei* is considered to be the major constraint to chickpea production. Twenty-one isolates of *A. rabiei* were obtained from blighted samples collected from major chickpea growing areas in Pakistan during 2006-08. Biological pathotyping on a set of chickpea differentials indicated that four isolates were highly virulent; two were less virulent, while rest of the isolates was moderately virulent. RAPD assay was used to investigate the genetic diversity among the isolates. Twenty primers with random nucleotide sequences were tested. A total of 49 bands were polymorphic. Dendrogram constructed from RAPD data indicated that there was a high genetic variability among the isolates collected from different locations and were grouped into three main clusters.

Key words: *Ascochyta rabiei*, blight, chickpea, DNA, Pathotypes, Genotyping

INTRODUCTION

Chickpea (*Cicer arietinum* L.) is the third most important pulse crop in the world, while it ranks first in the Indian subcontinent (Sarwar *et al*., 2012; Shah *et al*., 2013). It is one of the major protein sources in developing countries such as Pakistan and grows even on poor, sandy soils. Chickpea productivity, however, remained virtually stagnant over recent decades because of its susceptibility to diseases such as Ascochyta blight, Fusarium wilt, Neocosmospora root rot, Macrophomina charcoal/collar rot and phyllody (Akhtar *et al*., 2009; Ali *et al*., 2011; Chen *et al*., 2011).

Ascochyta blight caused by *Ascochyta rabiei* (Pass) Labrousse (teleomorph, *Didymella rabiei* (Kovachevski) v. Arx.), is the most important constraint to chickpea (*Cicer arietinum* L.) production in the World and often results in high yield losses (Chongo and Gossen, 2001; Ali *et al*., 2012b). The primary sources of inoculum are infested seed and stubble, but airborne ascospores play an important role in dispersal of the pathogen (Armstrong *et al*., 2001). Blight disease occurred in frequent epiphytotics in the northern parts of Pakistan since its first report in 1911 (Butler, 1918). Epiphytotic conditions prevail when virulent inoculum, favorable environmental conditions and susceptible host plants coexist (Jamil *et al*., 2000). It has been reported that severe epidemics of Ascochyta blight have occurred in various chickpea producing regions, including those regions where Ascochyta-resistant cultivars have been adopted (Navas-Cortes *et al*., 1998).

The analysis of genetic variation in pathogen populations is an important prerequisite to understand the co-evolution of plant-pathosystems. Variation in virulence is, however, not necessarily reflecting genetic variation (McDonald *et al*., 1989). Random amplified polymorphic DNA (RAPD) is a technique that is sensitive, quick to perform and can be applied to a large number of samples (Sarwar *et al*., 2000; Tonk *et al*., 2011; Ramessur *et al*., 2011). RAPD has become popular and used extensively for identification or differentiating strains of chickpea pathogens such as *Fusarium oxysporum* f.sp. *ciceris* (Kelly *et al*., 1994; Ali *et al*., 2012a), *Ascochyta rabiei* (Fischer *et al*., 1995; Jamil *et al*., 2010;). Molecular techniques have been used in several studies to investigate genetic diversity in *A. rabiei*, the relationship between molecular markers and pathotypes, and phylogenetic relationships (Udupa *et al*., 1998; Khan *et al*., 1999; Santra *et al*., 2001).

As the pathogens are changing constantly in nature and resistant varieties become susceptible after sometime, it is necessary to identify the strains of pathogen present/prevalent in particular region for the evaluation of new resistant varieties. The objectives of this study were to characterize *A. rabiei* isolates, collected from various areas of Pakistan, by biological pathotyping and genotyping for identification of aggressive isolates and their distribution in chickpea growing areas, which can be used for screening of chickpea germplasm to evaluate resistant varieties.

MATERIALS AND METHODS

Collection of blighted samples: Blighted plant tissues were collected from chickpea growing areas of Northern Punjab, NWFP and NARC Islamabad during 2006-08, when disease reappeared after several years.

Isolation and purification of *A. rabiei* from blighted samples: Isolation of the pathogen from diseased samples and its purification were done using standard

phytopathological techniques. Samples after sun drying were cut into 1-2 cm long pieces and plated on acidified potato dextrose agar (PDA) medium (2% of potato starch, dextrose and agar containing 7 ml^{-1} of 25% lactic acid) after surface sterilization with sodium hypochlorite for 1 min. Fungal growth was observed after one week incubation at 25ºC and after repeated streaking on CSMA medium (chickpea seed meal agar; 2%) until complete isolation and purification. Isolates were single spored and preserved on gram meal slants at -80ºC for further studies (Jamil *et al*., 2000).

Biological pathotyping: Twenty one *A. rabiei* isolates were tested for virulence using a host differential set comprising two susceptible (Aug-424, Pb-1), two tolerant (Aug-480, CM-72) and two resistant (Paidar, CM-88) chickpea cultivars. The experiment was conducted in earthen pots (3 pots/variety/isolates) in field environment.

Fungus culture and inoculation of plants: Mass culture of each isolate was multiplied on boiled, autoclaved chickpea seeds incubated at 20 ± 2ºC for about ten days until all the grains were fully covered with fungal spores. Spore suspension of each isolate was adjusted to10^6 spores/ml using a haemocytometer, one drop of Tween-20 per 500 ml suspension was added as a wetting agent and sprayed on each set of plants at flowering stage. Set of control plants was sprayed with sterilized water only. Plants were kept covered with wet coarse cloth under a big iron cage for 72 hours after inoculation. These plants were sprayed with a fine mist of water three times a day to keep the humidity high (above 90%) and to ensure maximum disease development. Disease severity was recorded 12 days after inoculation using a 1-9 rating scale described by Weising *et al.,* (1991), where 1 = no lesions; 2 = small tissue depression or spot; 3 = elongating spot; 4 = coalescent spot; 5 = stem girdling; 6 = stem breaking; 7 = lesion growth downward from breaking point; 8 = whole plant nearly dead and 9 = plant dead. Virulence data interpreted as 0.0-0.9 = avirulent (AV), 1.0-3.5 = low virulent (LV) 3.6-5.0 = medium virulent (MV), 5.1-9.0 = highly virulent (HV). Experiment was repeated two times.

Extraction of DNA and RAPD assay: Selected isolates of *A. rabiei* were processed for DNA extraction according to Jamil *et al*., (2010). For RAPD analysis preliminary twenty 10-mer oligonucleotide primers were tested to identify polymorphism. Nine primers were selected for further analysis based on the polymorphisms and banding patterns obtained after amplification. DNA amplification reaction was performed in a volume of 20µl containing DNA 1µl (10ng/µl), primer 4µl (5pmol/µl), PCR buffer 2µl, $MgCl_2$ 1.35µl (1.6mM), Taq DNA polymerase (Fermentas) 0.3µl (5µ/µl), dNTPS 10µl (0.5m M each of dATP, dTTP, dCTP and dGTP), H_2O 0.35µl. Amplification was performed with a thermocycler (Perkin-Elmer, 2400), programmed for 30 sec at 94ºC for preincubation, 35 cycles of 45 sec at 94ºC, 2 min at 33ºC, 2 min at 72ºC and final extension of 3 min at 72ºC. The reaction mixture without DNA template served as control and was electrophoresed along with the samples. Amplified DNA products were loaded onto 1.5% agarose gel containing 0.2mg/L ethidium bromide and electrophoresed in 1X TAE buffer. Gels were photographed under UV light.

Data Analysis: Comparison of each profile for each primer was done on the basis of the presence versus absence (1/0) of RAPD products of the same length. Bands of the same length were considered as identical. Analysis was done by UVITEC software and dendrogram was produced to calculate genetic distance values by cluster analysis.

RESULTS AND DISCUSSION

Pathogenicity of 21 selected *A. rabiei* isolates, from different chickpea growing areas, were tested using a host differential set comprising of susceptible (Aug424 and Pb-1), tolerant (Aug480 and CM72) and resistant (Paidar and CM88) varieties. Disease data recorded on each variety revealed that the aggressiveness of *A. rabiei* isolates was variable on host differential set (Table 1). Results revealed three distinct virulence groups i.e. highly virulent (HV), moderately virulent (MV) and less virulent (LV) as previously reported by Jamil *et al*. (2000, 2010) and Benzohra *et al*. (2010) in Pakistan and Northwest of Algeria, respectively. The possible existence of different races of *A. rabiei* was suspected due to variation in host-pathogen interaction and breakdown of host plant resistance in some cultivars (Jamil *et al*., 2010). Overall virulence calculated for each isolate indicated that 4 isolates were highly virulent, two of them belonged to National Agricultural Research Centre (NARC), Islamabad (I-79, I-80), one to Attock (A-50) and one to Peshawar (P-22). Aggressive isolates are thought to be evolved by mutation in response to a change in host resistance (Goodwill *et al*., 1994). Fifteen isolates belonging to Islamabad, Chackwal, Peshawar, Swabi and Mianwali (I-77, I-78, I-81, I-83, I-84, I-85, I-88, I-92, C-64, C-66, P-18, SB-1, SB-3, M-12, M-14) were moderately virulent and two isolates collected from NARC, Islamabad (I-76, I-82) were found less virulent. Isolates belong to less virulent group showed avirulence reaction on both resistant cultivars; were less virulent to tolerant varieties and medium virulent to susceptible ones. Moderately virulent isolates exhibited an avirulent to less virulent reaction on resistant varieties; a less to medium reaction on tolerant varieties and a medium to highly virulent reaction on susceptible varieties. *A. rabiei* isolates belonging to the highly virulent group were found less to medium virulent on resistant varieties;

medium to highly virulent to tolerant and susceptible varieties of the host differential set. Occurrence of less virulent *A. rabiei* isolates was very low, only found in the Islamabad area. The highest diversity was found in Islamabad where all three virulence groups were observed, this may be due to cool and wet climate which favours blight development. Since resistant chickpea germplasm from Pakistan and from outside this country such as from ICARDA and ICRISAT is screened there against Ascochyta blight disease. Field trials for screening resistant chickpea germplasm against blight were also conducted at Attock and Peshawar which may be the cause of pathogen mutation to highly aggressive group (Goodwill *et al*., 1994).

The biological pathotyping alone is not enough for a reliable identification and characterization of fungal pathotypes (Jamil *et al*., 2000). To identify genetic diversity of *A. rabiei* population of Pakistan RAPD analysis was conducted. RAPD analysis indicated that *A. rabiei* isolates were grouped together into three main clusters *i.e.* A, B and C (Fig-1). Five isolates were grouped in cluster A, six in B and ten in group C. A total of 52 loci were amplified with nine primers, of which 49 were polymorphic and 3 were monomorphic (Table 2). Primer G5 revealed the highest polymorphism on a set of 21 isolates (Fig-2). The dendrogram shows the relatedness and genetic distance among different isolates. Genetic similarity among 21 *A. rabiei* isolates belonging to different chickpea growing areas ranged from 10 to 85 %. The dendrogram shows that isolates C-64 and C-66 collected from the Chakwal area belonged to the same virulence group having the highest similarity (85%). Similarly SB-1 and SB-3 from Swabi belonged to moderately virulent showing 72% similarity. Isolates I-76, I-78, I-79, I-81 and I-77 collected from the same region of Islamabad having different virulence potential were grouped together in one cluster, while I-80, I-82, I-84, I-85 were fallen in another group. Two highly virulent isolates (I-79, I-80) from Islamabad showed 50% genetic similarity while I-76 and I-79 belonged to the less virulent and highly virulent groups having 80% homology. Other two highly virulent isolates (A-50, P-22) belonged to different areas and are genetically very different as these were grouped in two different clusters. Similarly two isolates from Mianwali M-12 and M-14 belonging to the same pathotype (moderately virulent) showed genetic diversity and were placed in separate groups. The results of the present study indicated that there was a vast genetic diversity among the *A. rabiei* isolates. This shows that isolates from the same area are genetically quite different and highly virulent or may be less virulent. This indicates the genetic diversity of *A. rabiei* isolates of different virulence within an area and between different areas (Jamil *et al*., 2010). Similar results have also been reported by Chang *et al*., (2008).

Table 1. Virulence data of *Ascochyta rabiei* isolates collected from major chickpea growing areas of Pakistan during 2006-08

Isolates	Area of collection	Differentials						Overall Virulence
		AUG-424	Pb-1	AUG-480	CM-72	Paidar	CM-88	
I-76	Islamabad	MV	MV	LV	LV	AV	AV	LV
I-77	Islamabad	HV	HV	MV	LV	MV	LV	MV
I-78	Islamabad	HV	HV	MV	LV	LV	LV	MV
I-79	Islamabad	HV	HV	HV	MV	MV	MV	HV
I-80	Islamabad	HV	HV	HV	MV	MV	MV	HV
I-81	Islamabad	HV	HV	MV	LV	AV	AV	MV
I-82	Islamabad	HV	MV	LV	LV	AV	AV	LV
I-83	Islamabad	HV	HV	MV	LV	LV	LV	MV
I-84	Islamabad	HV	HV	HV	LV	LV	LV	MV
I-85	Islamabad	HV	HV	MV	LV	LV	LV	MV
A-50	Attock	HV	HV	HV	MV	MV	LV	HV
C-64	Chakwal	HV	HV	MV	MV	LV	LV	MV
C-66	Chakwal	HV	HV	LV	MV	LV	LV	MV
I-88	Islamabad	HV	MV	MV	MV	LV	MV	MV
I-92	Islamabad	HV	MV	HV	MV	LV	LV	MV
P-18	Peshawar	HV	HV	MV	LV	LV	LV	MV
P-22	Peshawar	HV	HV	HV	HV	MV	LV	HV
SB-1	Swabi	HV	HV	MV	MV	LV	LV	MV
SB-3	Swabi	HV	MV	HV	MV	LV	LV	MV
M-12	Mianwali	HV	HV	MV	MV	LV	LV	MV
M-14	Mianwali	HV	MV	MV	HV	LV	MV	MV

AV = a virulent (0.0-0.9), LV = less virulent (1.0-3.5) MV = moderately virulent (3.6-5.0), HV = highly virulent (5.1-9.0)

They found that there is a high degree of genetic variation among the Canadian population of *A. rabiei* isolates. Several previous studies investigating the genetic diversity in the pathogen have examined the relationship between molecular markers and pathotypes (Weising *et al*., 1991; Fischer *et al*., 1995; Khan *et al*., 1999). Earlier studies on *A. rabiei* isolates showed that, pathogenicity data of *A. rabiei* isolates had no relationship to the genotyping results (Ahmad *et al*., 2007).

Although assessment of pathogenicity provides some classification information, but virulence data alone may not reflect the true genetic variability and evolutionary history of the isolates investigated. For example, isolates that are genetically distinct may have similar or identical virulence because they have been subjected to the same selection pressure by a common set of hosts (Zhang *et al*., 2003).

In the Present study we compared pathogenicity with genotypes in an *A. rabiei* population in Pakistan, which helped to identify a representative set of virulent isolates from different areas to make the screening of chickpea germplasm more reliable and durable. Knowledge of the variability of *A. rabiei* is also a prerequisite for breeding programmes aimed at obtaining durable resistance to *Ascochyta* blight (Pande *et al*., 2005; Ali *et al*., 2009). Reappearance (after 4-5 years) of Ascochyta blight indicated that the disease still persists as a major challenge to sustainable crop production. It also confirmed the existence and viability of the inoculum which can result in disease epidemic under highly conducive environmental conditions. It has been suggested that monitoring an *A. rabiei* population is a continuous study as evaluation of chickpea germplasm against prevailing strains of *A. rabiei* is prerequisite for obtaining durable resistant varieties.

Table 2. Primers used for generating random amplified polymorphic DNA patterns from 21 isolates of *Ascochyta rabiei*.

Primer	Nucleotide Sequence '5 to 3'	Polymorphic Bands	Monomorphic Bands
G-5	5'>GTGAGGGGTC<3	11	1
G-2	5'>AGGTTGCAGG<3'	4	0
G-4	5'>CTCCTGCCAA<3'	3	0
G-6	5'>TGAGCGGACA<3'	6	0
G-7	5'>TTGGCACGGG<3'	6	2
G-13	5'>AAGAGAGGGG<3'	6	0
G-14	5'>AAGGCGGCAG<3'	4	0
O-15	5'>GCAGAGAAGG<3'	5	0
O-16	5'>ACGGTGCCTG<3'	4	0

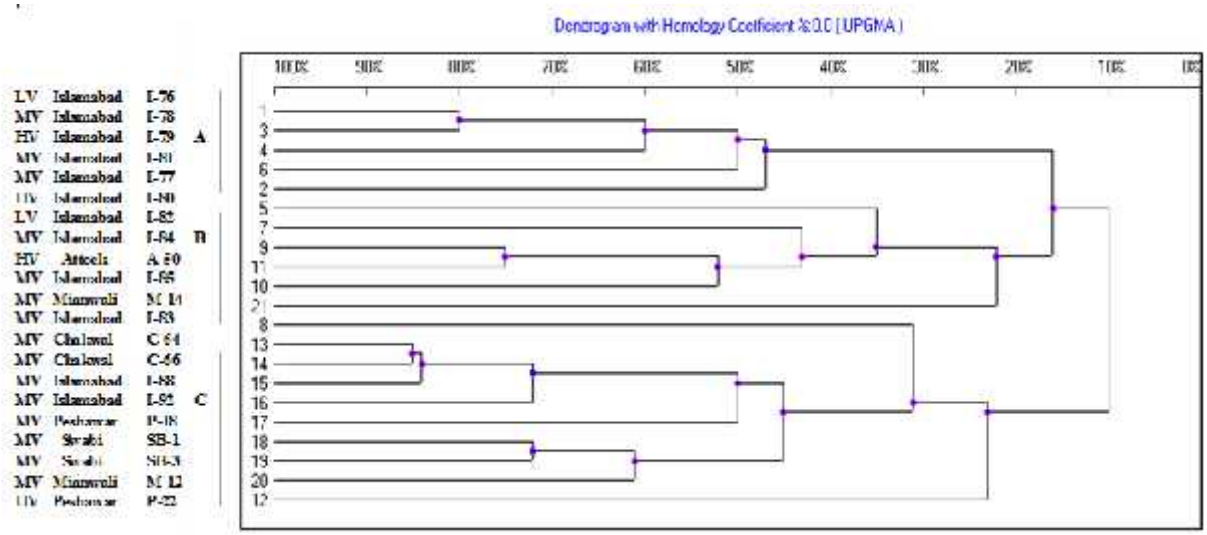

Fig 1. Dendrogram of 21 *A. rabiei* obtained from RAPD analysis using the un-weighted pair group method of arithmetic means (UPGMA) cluster analysis. The figure also shows the virulence and area of collection of isolates.

PrimerG-5: 5'>GTGAGGCGTC<3'

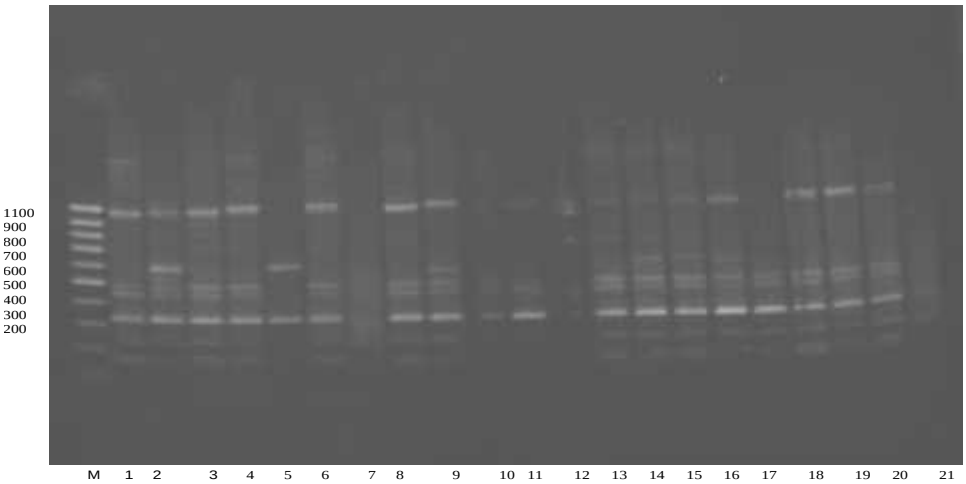

Fig. 2. Representative electrophoretic analysis of RAPD products of 21 *A.rabiei* isolates after amplification with the G-5 and G-14 primers.

M: 100 base pair DNA ladder

Lane 1 to 21: I-76, I-77, I-78, I-79, I-80, I-81, I-82, I-83, I-84, I-85 (Islamabad), A-50 (Attock), P-22 (Peshawar), C-64, C-66 (Chakwal), I-88, I-92 (Islamabad), P-18 (Peshawar), Sb-1, Sb-3 (Swabi), M-12, M-14 (Mianwali).

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