Pak. J. Bot., 42(2): 1369-1378, 2010.

GENOTYPING WITH RAPD MARKERS RESOLVES PATHOTYPE DIVERSITY IN THE ASCOCHYTA BLIGHT AND FUSARIUM WILT PATHOGENS OF CHICKPEA IN PAKISTAN

FARHAT F. JAMIL[*], MUHAMMAD SARWAR[1], NIGHAT SARWAR[1], JUNAID A. KHAN[1], M. HAYAT ZAHID[1], SUMAIRA YOUSAF[1], HAFIZ M. IMRAN ARSHAD[1] AND IKRAM-UL-HAQ[1]

Department of Botany, G.C. University, Faisalabad,
[1]Nuclear Institute for Agriculture and Biology (NIAB), P.O. Box 128, Jhang Road, Faisalabad. Pakistan.

Abstract

Chickpea (*Cicer arietinum* L.) is an important legume in Pakistan and constitutes about 70% of the pulse crops. Unfortunately, this crop is badly affected by two diseases i.e., chickpea blight and wilt caused by the fungi *Ascochyta rabiei* and *Fusarium oxysporum* f. sp. c*iceris* (FOC), respectively. Pathogenicity of *A. rabiei* and FOC isolates collected from different areas was conducted on a set of chickpea differentials sets. Isolates of the two pathogens showing different levels of virulence were selected for their DNA fingerprinting. RAPD analysis of 12 isolates of *A. rabiei* and 5 of FOC was performed using sixteen different 10-mer oligonucleotide primers. The dendrograms of *A. rabiei* and FOC produced from this data showed different levels of genetic relatedness and differences between isolates which were superimposed by their pathotyping data. A set of 7 genetically different and highly aggressive isolates of *A. rabiei* from different areas was selected for thorough screening of chickpea germplasm against blight for attaining durable resistance. Similarly, the dendrogram of FOC showed that all the five isolates were very different and those from Thal area were more aggressive.

Introduction

Chickpea (*Cicer arietium* L.) is the most important pulse crop in the world, but it ranks first in the Indian subcontinent and in the Mediterranean basin (Anon., 1994). It is one of the major sources of plant protein in developing countries such as Pakistan, and grows even on poor, sandy soils. Therefore, it is a crop of dry, marginal land in Asia, Africa and Central and South America. Chickpea is not only an important source of human food and animal feed, but it also fixes nitrogen, therefore, helps in the management of soil fertility, particularly in dry land areas (Sharma & Jodha, 1984).Chickpea is the most important grain legume in Pakistan, as it constitutes about 70% of the pulse crops. It occupies more than one million hectares (1,106,800 ha) with an average yield of 429 kg/ha (Anon., 2008). Sandy or sandy loam soils in non-irrigated areas in each of the four provinces of Pakistan represent its primary cropping sites, maximum being in Punjab (9, 89,000 ha). Thus, chickpea ranks among the prime crops in Pakistan for practical and cultural reasons (Jamil *et al*., 1997).

Many factors contribute towards less than optimal production of Chickpea in Pakistan. The most important factors that limit production of the crop include its susceptibility to disease and the lack of high yielding varieties. This crop is badly affected by two important and yield limiting diseases i.e., chickpea blight and wilt caused by the fungi *Ascochyta rabiei* and *Fusarium oxysporum* f.sp. *ciceris* (FOC), respectively.

[*]Corresponding author E-mail: farjamil153hotmail.com

Ascochyta blight is considered the major constraint to chickpea production in Pakistan. The disease causes substantial losses in yield especially in areas where conditions of high relative humidity (90 –95%), rainfall and low temperatures (20^{o}C) prevail during the growing season. Under epiphytotic conditions the crop is often totally lost (Hafiz, 1986). Quantitative information on the temporal and spatial distribution of *Ascochyta* blight from initial infection and the intensity of subsequent epiphytotics for cultivars with diverse response to the pathogen is crucial (Kimber *et al*., 2007) The disease was first reported in Pakistan in the North West Frontier Province in 1911 and since then has been appearing epidemically every 5-8 years in Pakistan where the Epidemic of 1979-80 alone produced a loss of US$ 90 million (Malik & Tufail, 1981). Unfortunately, those who suffer immediately from such crop losses are poor farmers with small or marginal land holdings. Due to this problem the farmers in the Barani Areas; in upper Punjab like Chakwal, Attock, Talagang and some parts of Thal where 70% of the crop is cultivated have switched over to grow other crops to avoid these substantial losses even that do not fit in those ecological conditions.

The second important disease is wilt caused by a soil borne pathogen FOC which can persist in the soil for many years without the presence of active host and has eroded chickpea crop from irrigated belts of Pakistan. Fusarium was first reported by Padwick (1940) and it developed in irrigated tract so badly that farmers have completely abandoned the cultivation of this crop. The share of chickpea cultivation was reduced from 50% during 1950's to 10% or less during 1990's. No serious effort was made to combat this menace till the release of varieties (CM-88 and CM-98) during 1990's. The application of fungicides is difficult in case of soil borne pathogens since it also destroys the normal flora of soil and in case of recontamination the pathogen flourishes faster due to the absence of competitive microflora and presence of susceptible host. This sometimes leads to higher disease incidence than without application of fungicides.

Pathogenic variability has been reported in both the pathogens (Porta Puglia, 1992; Jamil *et al*., 1995; Haware & Nene, 1982; Jimenez-Diaz *et al*., 1989). The pathogens are constantly changing in nature and making the resistant varieties susceptible after sometime. Therefore, continuous studies on characterization and geographic distribution of virulent races/strains of the pathogens and screening germplasm against them are inevitable to identify resistant germplasm. In addition, lack of high yielding varieties and chemical methods to control major diseases, in built genetic resistance is the only answer.

Many sources of resistance to *Ascochyta* blight and FOC wilt (Buksh *et al*., 2007) have been reported mainly based either on field observations during natural epidemics or on artificial inoculation either in the field or green house. Host resistance, however, does not persist as varieties presumed to be blight and wilt resistant failed, either as a result of genetic breakdown or a change in the virulence of the pathogen (Saxena & Singh 1987), examples of such cultivars includes the former "blight- resistant" lines/varieties F8, C12/34, C612, C727, C235, and Aug480. Varieties CM72 and C44, released later (1983) and exhibiting tolerance towards most of the known pathogenic strains of *A. rabiei*, also showed some level of susceptibility to few highly virulent strains (Jamil *et al*., 1993). CM-88 (1994), CM-98 & Bittal 98 (1998) and CM-2000 (2000) were released later as blight and wilt resistant varieties for general cultivation.

It is, therefore, necessary to identify the prevalent races of the pathogens. Pathogenic groups of *A. rabiei* and FOC were determined earlier recording the severity of infection symptoms on different chickpea cultivars (Porta-Puglia, 1992, unpublished data). Our preliminary work also showed the pathogenic potential of *A. rabiei* and FOC. However, the classical biological pathotyping techniques alone are not enough for a reliable identification and characterization of fungal pathotypes and populations (Jamil *et al*., 2000).

The aim of the proposed studies was to identify pathogenic and genetic diversity of isolates in *A. rabiei* and FOC population of Pakistan. These studies would also help to identify the geographical distribution and prevalence of more aggressive genotypes of the pathogens. These isolates would be used for reliable and durable screening of chickpea germplasm, which will supplement breeder's efforts for evolving disease resistant and high yielding varieties of chickpea. Conventional screening by using diseased plant debris or even spore suspension of a mixture of isolates is not as reliable as screening against individual, virulent isolates. Moreover, the information about geographical distribution in combination with the meteorological data would be helpful for disease forecasting.

Materials and Methods

Survey of chickpea crop: Annual surveys of Chickpea growing areas in Thal region (Jhang, Khushab, Mianwali, Bhakkar), Northern Punjab (Chakwal, Attock, Rawalpindi) and some areas of NWFP including Peshawar were conducted during March-April for the last three years. Blight was prevalent in Chakwal, Islamabad, Fatehjang and Attock while wilt was common in Thal area. Diseased samples of chickpea blight (*Ascochyta rabiei)* and wilt (*Fusarium oxysporum* f. sp. *ciceris)* were collected.

Isolation and Purification of *Ascochyta rabiei* from blighted samples: Blighted stem pieces (1 cm long) were surface sterilized with 1% hypochlorite for 1 min. and plated on acidified PDA medium (2% of each potato starch, dextrose and agar containing 7 ml/L of 25%, lactic acid). Fungal growth near lesions was observed after 5-7 days of incubation at 20^{o}C. A small amount of fungus culture was picked and streaked on CSMA (Chickpea Seed Meal Agar; 2%) until complete isolation and purification. Isolates were single spored and preserved on gram meal slants at -80^{o}C (Jamil *et al*., 2000).

Host differential set: Host differential set used for these studies comprised of six chickpea cultivars including Aug-424, Pb-1 (Susceptible), Aug-480, Paidar (tolerant) CM-72 and CM-88 (Resistant). Seeds were sown in plastic pots (2 pots/variety/isolate) filled with a homogenized mixture of clay and sand. Five plants were kept in each pot.

Preparation of mass culture: Boiled chickpea seeds were autoclaved in conical flasks (250 ml. cap.) and inoculated with spores of each isolate under aseptic conditions. The flasks were incubated at 20 ± 2^{o}C for about ten days until all the grains were fully covered with fungal spores.

Preparation of spore suspension: Mass culture of each isolate was shaken with 100 ml distilled sterilized water and filtered through muslin cloth. Spore concentration was adjusted to10^{6}spore/ml by using a Haemocytometer. A few drops of Tween 20 were mixed with spore suspension as a wetting agent before spraying on the plants.

Inoculation of plants: Two weeks old seedlings were sprayed with spore suspension to run off by using a plastic hand sprayer and covered with transparent plastic bags to maintain the humidity. The plants were kept under reduced light for 36 hours and after wards brought back to normal light after removing the covers. The plants were kept wet through sprinkling simple water several times a day until disease development.

Data recording: Disease data was recorded using a 9 Point Rating Scale (Weising *et al*., 1991) about two weeks after inoculation when maximum disease developed on susceptible varieties.

Isolation of FOC from wilted plants of chickpea: Roots of wilted plants collected annually from different chickpea growing areas were cut into 1cm long pieces especially from collar region, cut pieces were surface sterilized in 1% sodium hypochlorite for 1 minute and directly plated on Petri plates containing KM medium (Komada, 1975), which is a specialized medium for the isolation of *Fusarium oxysporum*. These plates were wrapped in polyethylene bags to avoid desiccation of medium, and then incubated in a cooled incubator (GallenKamp) at 25^{o}C in dark for 5 to 7 days. Fungal colonies appeared on KM medium were transferred on carnation leaf agar medium (Fisher *et al*., 1982) to confirm their identification because *Fusaria* produce stable characters on this medium. Identification was made by preparing slides in water and observed under microscope for the shape of conidia, conidiophores and formation of chlamydospores. These pure cultures were stored directly on CDA (Chickpea Dextrose Agar) in deep freezer (-20^{o}C) for further studies.

Pathogenicity/Virulence of FOC isolates: Each isolate was inoculated on boiled and sterilized chickpea seeds for the production of mass culture in 250 ml Erlenmeyer flasks. These flasks were incubated at 25^{o}C in dark for 8 to 10 days. Spore suspensions (mostly microconidia) were harvested by adding 100 ml sterilized water and shaking by hand for 5 minutes. Supernatant was filtered through 3 to 4 layers of muslin cloth to separate conidia from other materials like mycilium + chickpea seed coat. Conidia were adjusted $10^5 - 10^6$ spores/ml through haemocytometer. Chickpea varieties were grown in plastic pots (4" x 4" dia); filled with sterilized sand and were placed in the controlled temperature room at 25-28^{o}C, (illuminated with fluorescent and incandescent light). The four weeks old seedlings were harvested through washing the sand with tap water and their root portions were kept immersed in tap water. Roots of these plants were severed at about 1-2.5 cm from the root tip and then immersed in spore suspension for 3 hours. These plants were then transplanted in fresh plastic pots (4" x 4" dia) washed with Ethylalcohol and filled with sterilized sand. Sterilized tap water was applied after 2-3 days of interval depending on the requirement of plants. Plants were observed daily for the appearance of yellowing, dropping of upper and lower leaves and wilting. After 35-40 days of incubation in growth room, surviving plants were uprooted through washing with tap water and roots were kept submerged in a beaker until they were cut at cotyle and collar region to evaluate the degree of development of *Fusarium oxysporum* f.sp. *ciceris* (FOC) in the xylem vessels to evaluate resistance. Virulence of FOC isolates was determined on the basis of disease rating scale 1 based on degree of infection.

Scale 1 for Virulence of *Fusarium oxysporum* f.sp. *ciceris* (FOC).

Scale	Symptomology	Reactions
0.	No visible symptoms	Avirulent
1.	Few *Fusarium* blight lesions on secondary roots	Avirulent/Mild virulent
2.	Many *Fusarium* blight lesions on secondary roots, but no vascular discolouration	Mild virulent
3.	Many *Fusarium* blight lesions on secondary roots, with light vascular discolouration	Mild Virulent/Virulent
4.	Severe *Fusarium* blight lesions and prominent discolouration of vascular bundles	Virulent/Highly virulent
5.	Severe Fusarium blight lesions, complete discolouration of vascular bundles and wilting of plants	Highly virulent

Extraction of DNA: Selected isolates of *Ascochyta rabiei* and *Fusarium oxysporum* f. sp. *ciceris* were processed for isolation of DNA. Fifteen virulent isolates of *A. rabiei* and five isolates of *F. oxysporum ciceris* collected from different areas were selected from stock culture for this purpose. Isolates were grown on Czapek's Dox liquid medium in Roux bottles (1L capacity containing 100 ml of medium) for 15 days at 20 ^{0}C until a fresh weight about 15-20 g mycelia were harvested on filter papers. DNA was isolated according to a modified Cetyltrimethyl – Ammonium Bromide (CTAB) method (Weising *et al*., 1991). The fungal material was immediately shock freezed, ground under liquid nitrogen, dispersed in 125 ml 2 x CTAB extraction buffer (pre heated) at 60^{o}C and shaken in a 60^{o}C water bath for 30-45 min., an equal volume of chloroform: isoamylalcohal (24:1; v/v) was added, mixed well and centrifuged at 5000 rpm for 20 minutes. The upper phase was transferred to a fresh tube and nucleic acids were precipitated by adding 0.6 vol. of ice cooled isopropanol, and collected by centrifugation (5000 rpm, 4^{o}C; 20 min.), pellets were solublized in 750 ml TE buffer. DNA extracts were then treated with 10U of Rnase for 3-5 h at 37^{o}C. Rnase was removed by a phenol/chloroform extraction step. DNA was ethanol precipitated, centrifuged, dried and dissolved in an appropriate volume of TE buffer. The purified DNA was stored for RAPD analysis.

RAPD analysis: Sixteen different 10-mer oligonucleotide primers were tested on the DNAs of 12 *A. rabiei* and five FOC isolates to identify polymorphic amplicons. PCR was performed in 20 µl volumes containing 2 µl DNA (10 mg), 4 µl primer (20 mpol), 2 µl PCR buffer, 1.35 µl MgCl2 (2mM), 0.3 µl Taq DNA polymerase, 10 µl dNTPS (0.5 mM) each, 0.35 µl double-distilled water. Controls did not contain template DNA. Amplifications were performed on a Perkin-Elmer 2400 themocycler programmed for 30s at 94^{o}C (Denaturation), 35 cycles each of 45s at 94^{o}C , 2 min., at 40^{o}C, 2 min., at 72^{o}C, and final extension of 3 min., at 72^{o}C. The amplified products were loaded onto 1.5% agarose gels containing 0.2 mg 1^{-1} ethidium bromide and electrophoresed in 1x TBE buffer (40mM tris-acetate, 20 mM Sodium acetate, 1 mM EDTA, pH 7.8). The gels were viewed and their prints obtained by using gel documentation system.

Data evaluation: Data were compiled as a binary 0/1 Matrix by the presence (1) or absence (0) of a band at a particular position. Only major RAPD bands were considered for statistical analysis. Dendrograms were produced to calculate genetic distance values from simple matching of bands by cluster analysis using un-weighted pair group method of arithmetic means (UPGMA). Dendrograms of RAPD analysis of *A. rabiei* and FOC were superimposed by the biological pathotyping data to correlate the genetic distance in the pathogen isolates with their virulence.

Results and Discussion

Pathogenicity of *Ascochyta rabiei* isolates selected for RAPD analysis: The pathogenicity of 12 *A. rabiei* isolates including five from Islamabad (I-68, I-70, I-71, I-72, I-74) two from Chakwal (C-50, C-51), one from Attock (A-32), one from Mianwali (M-12) one from Peshawar (P-16) and two from Kaghan (KN-1 & KN-30) was conducted under controlled environment on a set of six chickpea differentials Aug-424 and Pb-1 (Susceptible), Aug-480 and Paidar (Tolerant), CM72 and CM88 (Resistant). Results showed that seven isolates (I-68, C-51, C-50, KN-30, A-32, P-16 and M-12) were

highly virulent, four isolates (I-70, I-71, I-72 and KN-1) were moderately virulent and only one isolate (I-74) was less virulent (Table 1). The possible existence of different races of *A. rabiei* was suspected because of the variation in host-pathogen interactions and breakdown of host plant resistance in some cultivars at different locations. 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). Management of *Ascochyta* blight is essential to provide increased and stable chickpea yields throughout the world.

Pathogenicity of FOC isolates: Pathogenicity/Virulence of five FOC isolates, collected from different areas, was tested against five Chickpea standard varieties used as differentials, Aug-424 (highly susceptible), Pb-1 and CM-2000 (tolerant) and C-727 and CM-98 (resistant). Virulence of these isolates varied against all the 5 varieties. On the basis of overall virulence, 2 isolates were highly virulent, 1 was virulent and 2 were mild virulent (Table 2). These five isolates having different virulence levels and belonging to different chickpea growing areas were selected for genotyping.

Table 1. Pathogenicity of *Ascochyta rabiei* isolates selected for RAPD analysis.

Isolates*	Differentials						Overall virulence
	Aug-424	Pb-1	Aug-480	PAIDAR	CM72	CM88	
I-74	HV	MV	LV	LV	LV	LV	LV
I-68	HV	HV	HV	HV	MV	MV	**HV**
C-51	HV	HV	HV	MV	HV	MV	**HV**
I-71	HV	HV	MV	LV	LV	LV	MV
I-70	HV	HV	MV	LV	LV	LV	MV
I-72	HV	HV	MV	LV	LV	LV	MV
KN-1	HV	MV	LV	MV	LV	LV	MV
KN-30	HV	HV	HV	MV	MV	MV	**HV**
C-50	HV	HV	HV	MV	HV	MV	**HV**
A-32	HV	HV	HV	MV	MV	LV	**HV**
P-16	HV	HV	HV	MV	MV	MV	**HV**
M12	HV	HV	HV	MV	LV	LV	**HV**

Rating scale: AV (Avirulent)= 0.0–0.9; LV (Low virulent)= 1.0–3.5; MV (Medium virulent)= 3.6–5.0; HV (Highly virulent)= 5.1–9.0.

***Area of collection:** Islamabad= I-68, I-70, I-71, I-72, I-74; Kaghan= KN-1, KN-30; Peshawar= P-16; Attock= A-32; Chakwal= C-50, C-51; Mianwali= M-12 (SS1)

Table 2. Pathopgenicity/virulence of *Fuarium oxysporum* f. sp. *ciceri* isolates selected for RAPD analysis.

S.#	Area	FOC isolates	Aug-424	C-727	Pb-1	CM-98	CM-2000	Overall virulence
1.	Grote	2202	HV	HV	HV	V	HV	HV
2.	Adhi Kot	2212	HV	V	HV	V	HV	HV
3.	Gowhar Wala	2216	V	MV	MV	MV	MV	MV
4.	D.I. KHAN	2215	V	V	V	V	V	V
5.	D.I. KHAN	2220	MV	V	MV	MV	V	MV

RATING SCALES: AV (Avirulent) = 0−0.9; MV(Mild Virulent) = 1−2.9; V(Virulent) = 3−3.9; HV(Highly Virulent)=4−5

Table 3. Primers showing polymorphism among *A. rabiei* and FOC isolates.

S.No	Primer	Nucleotide sequence
1.	Oligo 1	CAGCACCCAC
2.	Oligo 2	GGTGACGCAG
3.	Oligo 3	GTTAGTGCGG
4.	Oligo 4	ACGGTCTTGG
5.	Oligo 5	ACGATCGCGG
6.	Oligo 6	GAAACAGCGG
7.	Oligo 7	CTCGACACTG
8.	Oligo 12	GTCCTCAACG
9.	Oligo 13	CGTCGTTACT
10.	Oligo 14	CCTGATGACC
11.	Oligo 15	GCAGAGAAGG
12.	Oligo 16	ACGGTGCCTG

RAPD analysis: Out of 16 tested 10-mer oligonucleotide primers, the twelve primers (Table 3) revealed good polymorphism on a set of l2 selected *A. rabiei* and 5*Fusarium oxysporum* f.sp. *ciceris* isolates (Figs. 1A, B, C, D). Although amplification patterns differed, several amplification products were shared by all isolates, which proves well-adapted PCR conditions with high reproducibility. The pathogenicity data was superimposed on the dendrogram of *A. rabiei* isolates obtained from RAPD data to establish their bio and genetic diversity. The resulting dendrogram of *A. rabiei* isolates (Fig. 2) shows the relatedness and genetic distances among different isolates. Genetic distances ranged from 0.60 to 0.94. The dendrogram shows that there are two most closely related isolates as compared to others I-71 and I-72. Both are from Islamabad and both are mild virulent. They show many bands that bring them together, but still they are somewhat different and have a genetic distance of 0.60.

There are three highly virulent isolates from different areas, like P-16 from Peshawar, A-32 from Attock and C-50 from Chakwal showing a genetic distance of 0.65, while I-74 and I-68 showing virulence levels LV and HV, both from Islamabad show a genetic distance of 0.70. Similarly KN-1 and KN-30 from Kaghan showing MV and HV are only distantly related. Two isolates from Chakwal C-51 and C-50, both are highly virulent but are very distantly related (genetic distance 0.94). This shows that isolates from same area are genetically very 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 in different areas.

A set of 7 different highly aggressive isolates, was selected including 4 from Chakwal, one from Islamabad, one from Mianwali, one from Peshawar, one from Attock and one from Kaghan. This set will be used for thorough screening of chickpea germplasm against blight for attaining durable resistance. The *A. rabiei* isolates collected this year if proved genetically different from previous ones and highly aggressive will be included in this set in future.

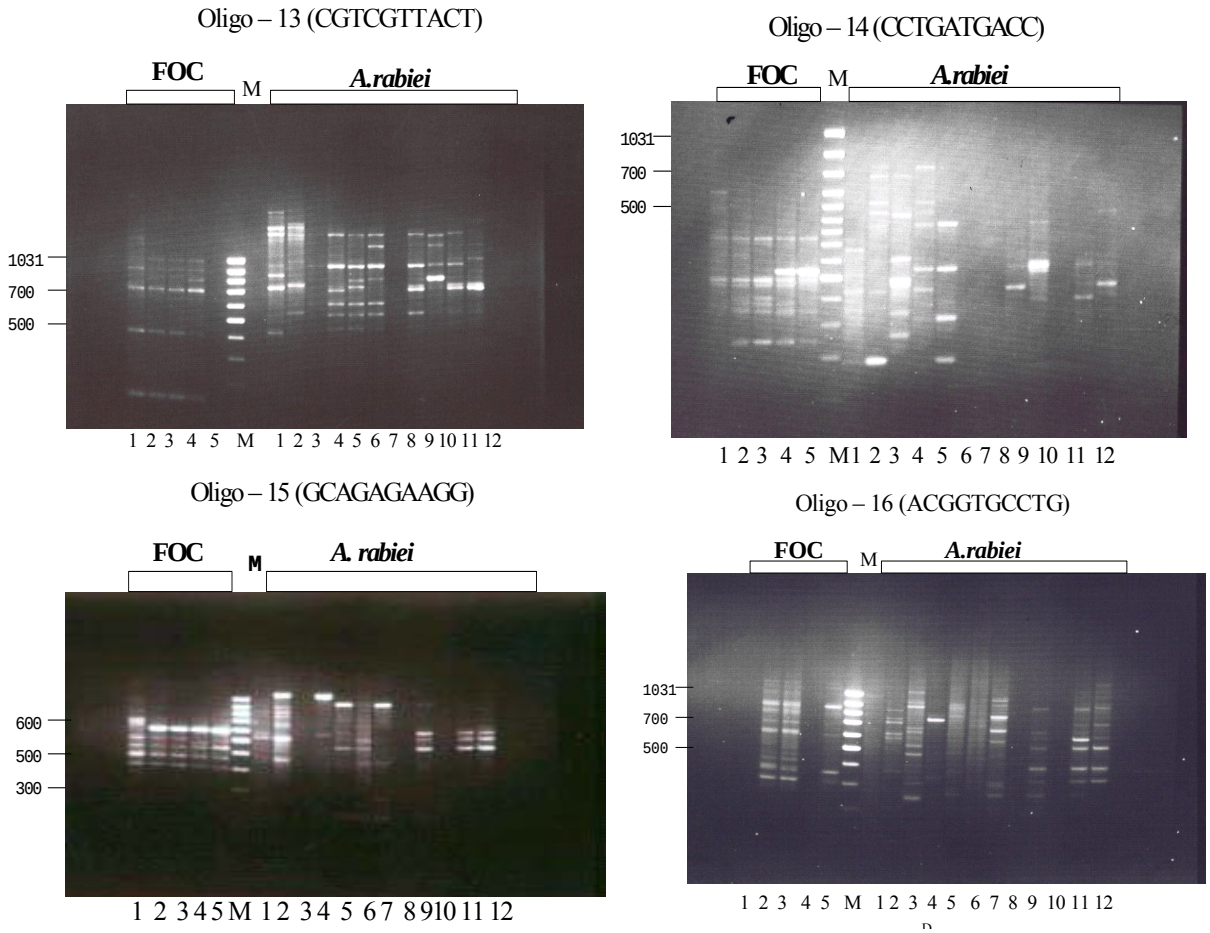

Fig. 1 Representative electrophoresis gels of *Ascochyta rabiei* and *Fusarium oxysporum ciceri* isolates after RAPD analysis with different primers.

FOC isolates;1(2220),2(2215),3(2216),4(2212),5(2202); *A. rabiei* isolates; 1(M-12), 2(P-16), 3(A-32), 4(C-50), 5(KN-30), 6(KN-1), 7(I-72), 8(I-70),9(I-71),10(C-51),11(I-68),12(I-74); M: Marker,100 base pair ladder

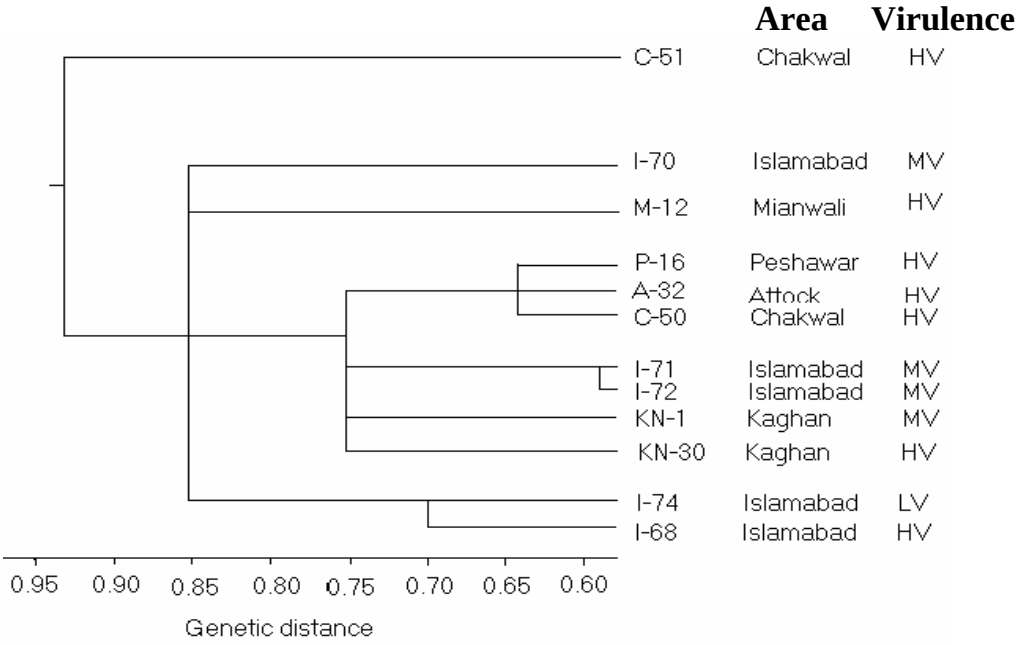

Fig. 2. Dendrogram of *A. rabiei* isolates developed from RAPD data obtained from different, 10-mer oligonucleotide primers using un-weighted pair group method of arithmetic means (UPGMA). Figure also shows the virulence and area of collection of isolates.

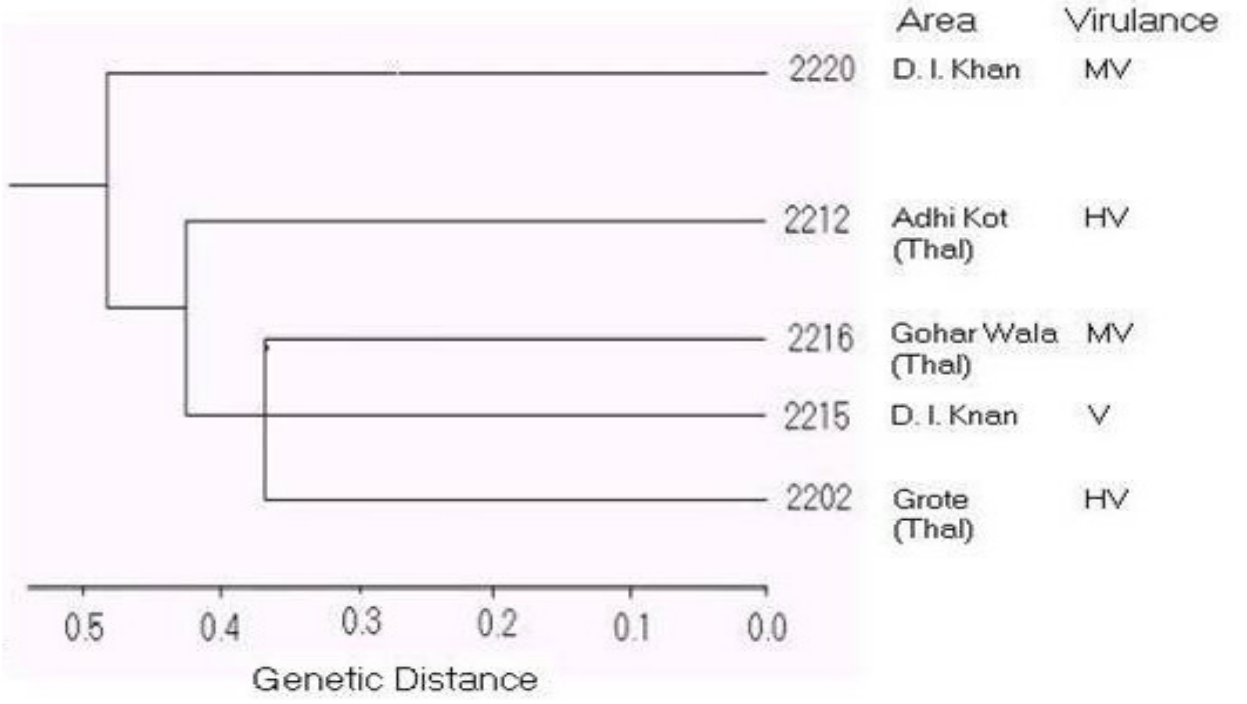

Fig. 3. Dendrogram of five FOC isolates developed from RAPD data obtained from different 10-mer oligonucleotide primers using un-weighted pair group method of arithmetic means (UPGMA). Figure also shows the virulence and area of collection of isolates.

Dendrogram of FOC (Fig. 3) showed that at 0.5 genetic distance the isolates were divided into three major groups. Isolate 2216 and 2202 having different virulence levels but belonged to Thal area were present in a same group, while another isolate, 2212 collected from the same area was fallen in another group. Two isolates belonged to D. I. Khan having different virulence levels fall in two different groups. Similar results were reported by Singh *et al*., (2006) on FOC isolates from India. All the five isolates were very different, moreover, the isolates from Thal area were found more aggressive/virulent. These studies are in continuation of our previous studies (Jamil *et al*., 2000). The highly virulent isolates collected after 1998 and which were not included in the previous studies were also included in this study to have a better screening of germplasm. Extent of high incidence of disease in an area predicts a high level of disease incidence if the favorable environmental conditions prevail in the next season and make us alert to adopt all the precautionary measures.

Acknowledgement

The authors acknowledge Pakistan Science Foundation for funding this research through grant No. PSF/RES/BIOTECH/P-NIAB/AGR (38).

References

Ali, S.R., S.M. Iqbal., U. Iqbal; A. Ghafoor and A. Akram. 2009. Pathogenic diversity in *Ascochyta rabiei* (Pass.) Lib., of chickpea. *Pak. J. Bot.,* 41(1): 413−419.

Anonymous. 1994. *Food and Agriculture Organization of the United Nations Organization*. Production Year-Book FAO, Rome, Italy.

Anonymous. 2007-08. *Agricultural Statistics of Pakistan*, Govt. of Pakistan. pp. 44 45.

Bukhsh, A., S.M. Iqbal and Ikramul Haq. 2007. Evaluation of chickpea germplasm for wilt resistance. *Pak. J. Bot*., 39(2) : 583−593.

Fisher, N.L., L.W. Burgess, T.A. Toussoun and P.E. Nelson.1982. Carnation leaves as a substrate and for preserving cultures of Fusarium species. *Phytopathol*, 72: 151-153.

Hafiz, A. 1986. *Plant Diseases*. Directorate of Publications. Pakistan Agricultural Research Council, Islamabad.

Haware, M.P. and Y.L. Nene. 1982. Races of *Fusarium oxysporum* f.sp. *ciceri*. *Plant Disease*, 66: 809-810.

Jamil, F.F., M. Sarwar, I. Haq and N. Bashir. 1993. Pathogenic variability in *Ascochyta rabiei* causing blight of chickpea in Pakistan. *International Chickpea Newsletter*, 29: 14-15.

Jamil, F.F, M. Sarwar, I. Haq and N. Bashir. 1995. Identification of pathotypes in *Ascochyta rabiei* (Pass.) Lab., the cause of chickpea blight in Pakistan. *Pakistan Journal of Botany*, 27(1): 193-199.

Jamil, F.F., I. Haq, M. Sarwar and N. Bashir. 1997. Characterization of pathotypes population in *Ascochyta rabiei* (Pass.) Lab., the cause of chickpea blight in Pakistan. *Proceedings of the Fourth International Symposium Workshop* on "The application of Molecular Biological Research in Agriculture, Health and Environment" (Ed.): S. Riazuddin. National Centre of Excellence in Molecular Biology, Lahore 8-11 April, 1995, pp. 145-148.

Jamil, F.F., N. Sarwar, M. Sarwar, J.A. Khan, J. Geistlinger and G. Khal. 2000. Genetic and pathogenic diversity within *Ascochyta rabiei* (Pass.) Lab., population in Pakistan causing blight of chickpea (C*icer arietinum* L.). *Physiol and Molecular Plant Pathol*., 57: 243-254.

Jimenez-Diaz, R.M., A. Trapero-Casas and J. Cabrrera de La Colina. 1989. Races of *Fusarium oxysporum* f.sp. *ciceri* infecting chickpea in South Spain. In: *Vascular wilt diseases of plants*. (Eds.): E.C. Tjamos and C. Beckman. NATO AS/Series, Vol. H28, Berlin, Germany: Springer-Verlag.

Kimber, R.B.E., D. Shteinberg, M.D. Ramsey and E.S. Scott. 2007. The role of seedling infection inepiphytotics of *Ascochyta* blight of chickpea. *Eur. J. Plant Pathol*., 117: 141-152.

Komada, H. 1975. Development of a selective medium for quantitive isolation of *Fusarium oxysporum* from natural soil. *Review of Plant Protection Research*, 8: 114-125.

Malik, B.A. and M. Tufail. 1981. Chickpea production in Pakistan. In: *Proceedings of the workshop on Ascochyta blight and winter sowing of chickpeas*. (Eds.): M.C. Saxena, K.B. Singh. ICARDA, Allepo, Syria, 4-7 May. 1981, pp. 229-235.

Padwick, G.W. 1940. The genus *Fusarium*. III. A critical study of the fungus causing wilt of gram (*Cicer arietinum* L.) and of the related species of the sub-section Orthocera, with special relation to the variability of key characteristics. *Indian J. Agric. Sci*., 10: 241-248.

Pande, S., K.H.M. Siddique, G.K. Kishore, B. Bayaa, P.M. Gaur, C.L.L. Gowda, T.W. Bretag and J.H. Crouch. 2005. *Ascochyta* blight of chickpea (*Cicer arietinum* L.) a review of biology, pathogenicity and disease mangement. *Australian J. Agri. Res*., 56: 317-332.

Porta-Puglia, A. 1992. Variability in *Ascochyta rabiei*. In: *Disease Resistance Breeding in Chickpea*. (Eds.): K.B. Singh & M.C. Saxena. Proceedings of the consultative meeting on breeding for disease resistance in Kabuli chickpea. 6-8 March, 1989. ICARDA, Aleppo, Syria. pp. 135-143.

Saxena, M.C. and K.B. Singh. 1987. *The chickpea*. C.A.B. International, Walling ford Oxon, U.K.

Sharma, D. and N.S. Jodha. 1984. Pulse production in Semi-arid region of India. *Proceedings of pulses production, constraints and opportunities*. pp. 241-265.

Singh, B.P., R. Saikia, M. Yadav, R. Singh, V.S. Chauhan and D.K. Arora. 2006. Molecular characterization of *Fusarium oxysprum* f. sp. *ciceri* causing wilt of chickpea. *African J. Biotechnology*, 5: 497-502.

Weising, K., D. Kaemmer, J.T. Epplen, F. Weigand, M. Saxena and G. Khal. 1991. DNA fingerprinting of *Ascochyta rabiei* with synthetic oligonucleotides. *Current Genetics*, 19: 483-489.

(Received for publication 11 June 2009)