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ISOZYME POLYMORPHISM IN *ASCOCHYTA RABIEI* ISOLATES FROM PAKISTAN

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Abstract

Fifteen isolates of *Ascochyta rabiei* causing blight of chickpea (*Cicer arietinum*) were collected from Pakistan and analyzed for esterase, acid phosphatase, alcohol dehydrogenase, lactate dehydrogenase, malate dehydrogenase, glutamate dehydrogenase and fumarase. Only two enzymes viz., esterase and acid phosphatase produced qualitative as well as quantitative differences among the isolates of *A. rabiei*. Six electrophoretic phenotypes were developed in case of acid phosphatase, whereas isoelectric focusing of the protein extracts revealed simpler banding patterns for the esterase activity on gel. No correlation between isozyme patterns and the pathogenic groups were established on the basis of aggressiveness.

Introduction

Pathogenic variability of *Ascochyta rabiei* continues to pose a major problem for plant breeders in the development of durable forms of blight resistance in chickpea (Bedi & Aujla, 1969; Kaiser, 1973; Nene & Reddy, 1987; Porta-puglia *et al.*, 1985; Hussain & Malik, 1991). The classical biological pathotyping technique, using a set of different host genotypes (Reddy & Kabbabeh, 1985), is laborious, time consuming and requires strict standardization of test conditions. Molecular markers are useful tools for examining genetic variation within populations of phytopathogenic fungi (Michelmore & Hulbert, 1987).

There are several molecular techniques available for studying the genetic relationships of fungal populations viz., Restriction Fragment Length Polymorphism (McDonald & Matinez, 1990), DNA sequence divergence (Schardl *et al.*, 1991), DNA fingerprinting (Levy *et al.*, 1991) and Polymerase Chain Reaction with arbitrary primers (Welsh & McClelland, 1990; Williams *et al.*, 1990) which have been used successfully in the study of fungal populations. In recent past, analysis of isozyme variation have also been extensively used in plant and animal species and only recently they were employed with pathogenic fungi (Burdon *et al.*, 1983; Burdon & Roelfs, 1985; Leung & Williams, 1986; Levy *et al.*, 1991; Tooley *et al.*, 1985; Michelmore & Hulbert, 1987). This technique has also been used as a taxonomic tool to differentiate closely related phytopathogenic fungi with distinct host ranges (Bosland & Williams, 1987; Liu *et al.*, 1990).

In the present studies isozyme polymorphism was used as a marker to determine variability among various isolates of *A. rabiei* collected from different regions of Pakistan. There does not appear to be any previous report in which isozyme analysis was used on this fungus.

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Materials and Methods

Plant growth: Seeds of chickpea cultivars C-727, C-44, CM-72, ILC-263, Noor-91, Paidar-91 and Punjab-91 were obtained from Pulses Programme, National Agricultural Research Centre, Islamabad, Pakistan. Five plants of each genotype were grown in iron trays with two replications. The environmental chamber was supplied with a photo period of 12 hrs per day. Light was generated by four fluorescent tubes and three day-light bulbs. Temperature was maintained at 16°C during 12 h night and 25°C during 12 h daytime.

Preparation of single culture: Isolates of *A. rabiei* were immersed in sterile distilled water to release the spores. The resulting spore suspensions were dispersed on water agar and incubated at 20°C to induce germination. After 48 h of germination, single spores were transferred onto the fresh chickpea seed meal agar medium for further culture.

Pathogenicity test: Autoclaved chickpea grains were inoculated by *A. rabiei* to obtain spore suspension (Alam *et al.*, 1987). Ten days infected grains were suspended in sterile distilled water for the collection of spores. Spore suspensions containing 100,000 spores/ml were sprayed on 7 day old seedlings and incubated for 5 days at 20 ± 2°C. The plants were sprayed with water twice a day for maintaining humidity. Results of disease severity on single plants were taken from the 5th day till the 10th day after inoculation using the scale after some modification (Weising *et al.*, 1991; Reddy & Singh, 1984; Singh *et al.*, 1981). Infection % was obtained by using the following formula:

$$\text{Infection \%} = \frac{\text{Sum of all disease ratings x 100}}{\text{No. of infected plants x maximum disease rating}}$$

Estimation of dry weight of mycelium: About one million spores of 10 days old cultures were inoculated in Richard liquid medium and incubated in Model G-25 incubator shaker (New Brunswick Scientific Co. Edison, New Jerssey, USA) at 125 rpm and at 20°C. Mycelium was harvested from different flasks at 2,3,4,6 and 8 days intervals and lyophilized for dry weight determination.

Extraction of proteins from mycelium: Proteins were extracted by grinding the lyophilized mycelium in pestle and mortar using extraction buffer: 0.1M Tris-HCl, pH 7.0; 10 mM KCl; 0.1mM $MgCl_2$; 1mM EDTA-Na_2; 145mM B-mercaptoethanol and 0.1 mM ascorbic acid (Simcox *et al.*, 1992).

Estimation of protein: Crude extracts were passed through sephadex G-25M, PD-10 column (Pharmacia, Biotech) with destilled water. Protein concentration was analyzed (Bradford, 1976) with bovine serum albumin (Sigma) as a standard.

Determination of enzyme activity: Esterase activity was determined, using a reaction mixture containing 100 mM Na-phosphate buffer (pH 7.5) with 0.1% triton-100-X, 50mM p-nitrophenyl acetate dissolved in Methyoxyethanol and enzyme extract to final volume of 1 ml (Kohler & Barz, personal communication, Munster University, Germany). Acid phosphatase was estimated, in a reaction mixture containing 100mM Na-acetate buffer (pH 6.0) 10mM p-nitrophenyl phosphate dissolved in methoxyethanol and enzyme extract to final volume of 1 ml after some modifications (Tenhaken, 1992). The

other enzymes viz., fumarase (Walk & Hock, 1977), lactate dehydrogenase (Asker & Davies, 1984), alcohol dehydrogenase (Smith *et al.*, 1971), malate dehydrogenase (Hock, 1973) and glutamate dehydrogenase (Hartmann *et al.*, 1973) were tested according to the references.

Isoelectric focusing: Isoelectric focusing polyacrylamide gel was prepared on gel bond-page film (Pharmacia) containing acrylamide, N, N'methylenbisacrylamide (T=30%, C=3%), 0.25 M Tris-HCl pH 8.4, glycerin 87% (g/v), N,N,N^6,N^6 - Tetramethyl-ethylendiamin (Temed) and ammonium per sulphate (APS) 40% (g/v). The gel was dried overnight at room temperature and then rehydrated for 2 h with a mixture containing 10% sorbitol and 1.2 ml ampholine or pharmalyte 2.5% (g/v) (pH 3 to 10), placed on the slab at 10°C and run for 1/2 h at 2000 voltage and 14 watts with a constant cooling temperature of 10°C before applying the samples. Fifteen microgram protein was applied in two replications on filter paper (sample appl. piece) and electrophoresis was performed for another 1.5 hrs.

Enzyme activity was detected by incubating the gel in their required staining solutions. The gels were incubated in 50mg indoxy acetate dissolved in 1 ml methoxyethanol with the addition of 10ml 0.1M Na-phosphate buffer, pH 7.5 for the esterase (Kohler & Barz, personal communication, Munster University, Germany); 54 mg alpha-napthylacid phosphate and 20mg Fast garnet GBC in 20 ml, 100mM Na-acetate buffer, pH 6.0 for acid phophatase with certain modifications (Griffin & Palmer, 1987) for about 60-90 minutes and adding 2% acetic acid. Similarly the other enzymes activities like fumarase (Edwards & Hopkinson, 1979), lactate dehydrogenase (Poerio & Davies, 1980), alcohol dehydrogenase (Smith *et al.*, 1971), malate dehydrogenase (Schweiger *et al.*, 1967) and glutamate dehydrogenase (Hartmann *et al.*, 1973) were also tested on the gels.

Estimation of pH values across the isoelectric focusing gel: Electrophoresed gel without sample was cut into 1cmq. pieces starting from anode to cathode. Each piece was equiliberated with distilled water in a test tube and pH was measured using pH paper (Schweiger & Barz, Personal communication, Münster University, Germany).

Results

Pathogenicity test: For pathogenicity grouping 7 chickpea genotypes were inoculated with *A. rabiei* isolates collected from different chickpea growing areas in Pakistan. On the basis of disease infection percentage 4 groups were established i.e., less aggressive (less than 40% infection), moderately aggressive (40-60% infection), aggressive (60-80% infection) and highly aggressive (80-100% infection). Amongst the 15 randomly selected isolates 9, 4 and 2 were respectively, highly aggressive, aggressive and moderately aggressive (Table 1). No isolates were found in the less aggressive group.

Enzymes activity: Initially, 7 enzymes i.e., esterase (E.C. 3.1.1...), acid phosphatase (E.C. 3.1.3.2.), alcohol dehydrogenase (E.C. 1.1.1.1..), lactate dehydrogenase (E.C. 1.1.1.27.), glutamate dehydrogenase (E.C. 1.4.1.3.), fumarase (E.C. 4.2.1.2.) and malate dehydrogenase (E.C. 1.1.1.37.) were tested with protein extracts of 2 isolates to determine which enzymes showed qualitative and or quantitatively different isozyme patterns on the gels. Of these only 2 enzymes viz., esterase and acid phosphatase

Table 1. Isolates of *Ascochyta rabiei*, their codes, year of collection, infection % and pathogenicity reaction.

S.No.	Isolates code	Year of collection	Location	Infection %	Aggressiveness
1.	P-10	1986	Chakwal	47	Moderate
2.	P-11	1986	Chakwal	100	High
3.	P-13	1986	Chakwal	78	Aggressive
4.	P-CH	1986	Chakwal	100	High
5.	P-KN-1	1985	Kaghan	62	Aggressive
6.	P-2	1986	Kaghan	95	High
7.	P-26	1988	Kaghan	49	Moderate
8.	P-31	1985	Kaghan	93	High
9.	P-F.J	1986	Fateh Jang	95	High
10.	P-1	1988	Fateh Jang	92	High
11.	P-4	1987	Tarnab	100	High
12.	P-7	1987	Tarnab	62	Aggressive
13.	P-23	1987	Attock	100	High
14.	P-NC-1	1985	Islamabad	70	Aggressive
15.	P-39-R	1985	Kullorkot	83	High

produced qualitative as well as quantitative differences among isolates of *A. rabiei*. Glutamate dehydrogenase was the only enzyme tested that showed no band at all. Some other enzymes produced several bands viz., lactate dehydrogenase (3), fumarase (5) and alcohol dehydrogenase (6) whereas with malate dehydrogenase, 5 were found to show shaky bands which were excluded from further studies because they showed less desirable results and found to be monomorphic.

In all the isolates, cellular protein concentrations were higher after 3 and 4 days of growth with substantial decline after 6 days of growth. Similarly in case of acid phosphatase, the intensity of the bands on gels was higher using the protein extracts after 2 and 3 days of growth. Afterwards the intensity of the bands declined and in case of some isolates, several bands disappeared. Therefore, best result were obtained with the protein extracts after 2-3 days of growth.

An electrophoretic phenotype (EP) is defined as a group of isolates having banding patterns in common. In case of acid phosphatase, 6 different groups were found (Fig.1). EP-1 was differentiated from other EP groups on the basis of having only single anodic band at pH 4.7. In EP-2, no cathodic band appeared. EP-3 was differentiated from EP-4 on the basis of one missing cathodic band at pH 7.9 and EP-6 was differentiated from EP-5 having double cathodic bands in between pH 8.5-9.4. Maximum number of isolates were found in EP-5 and these isolates were preferentially found in the Kaghan area whereas the isolates from Chakwal area had to be placed in different EP groups indicating that the isolates from this area represent a mixed population (Table 2).

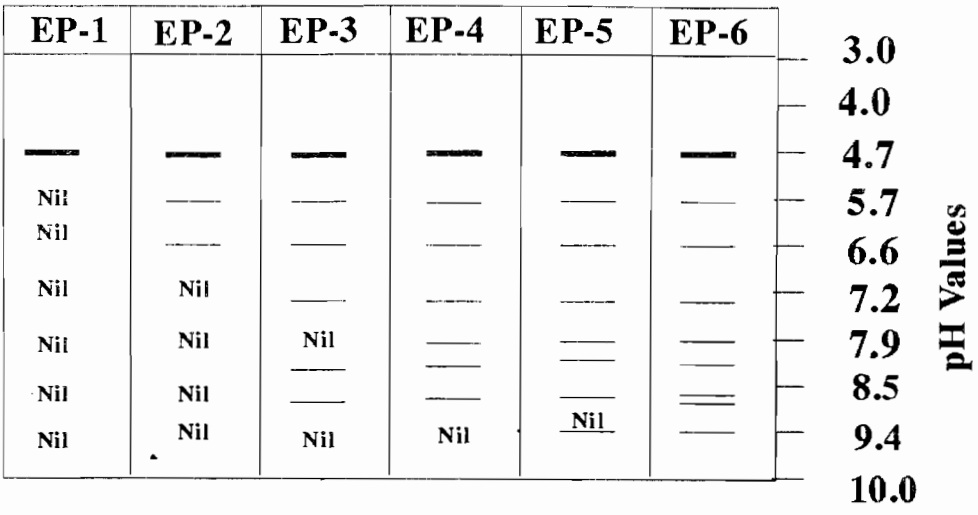

Fig.1. Diagramatic representation of electrophoretic phenotypes of acid phosphatase in *A. rabiei* isolates. Electrophoretic phenotype (EP) is defined as a group of isolates having banding patterns in common. Whole sets of isolates were run three times on gels with 2 replications.
EP = Electrophoretic phenotypes, Nil = No band appeared

It was also observed that the protein extracts of all isolates had several anodic (at pH 5.7 and 6.6) and cathodic bands (at pH 7.2 to 9.4) of acid phosphatase which completely disappeared or expressed with low intensity at later periods (i.e., 4 and 6 days) of mycelial growth. Furthermore, the major band of acid phosphatase (at pH 4.7) which has been detected in the protein extracts of all isolates during the total growth period also tended to be expressed with less intensity during later stages of the fungal growth.

In a highly aggressive and a moderately aggressive isolates, esterase activity (substrate: para-nitrophenyl acetate) measured in protein extract collected at different stages of fungal growth after 4 days of growth the activity was found to be 1.36 and 3.04 nkat/mg of protein, respectively, but it gradually declined upto the 8th day of growth. When esterase activity was tested on the gels (substrate: indoxyl acetate) bands of low and high intensity was consistently found with the 4 days protein preparation for the highly aggressive and moderately aggressive isolates. In general, isoelectric focusing of the protein extracts revealed simpler banding pattern for the esterase than that for acid phosphatase.

In another experiment, 4 day old mycelium used to test the protein extracts of 15 isolates showed that esterase activity varied in highly aggressive, aggressive and moderately aggressive isolates from 1.15-1.57 nkat/mg protein, from 1.26-3.7 nkat and 2.43-3.15 nkat/mg of protein, respectively. Again on the gel also different intensity of bands were observed. On the basis of different intensities of bands 3 electrophoretic phenotypic groups were established, low intensity band at pH between 4.0-4.7 (EP-1), high intensity band at pH between 4.0-4.7 (EP-2) and low intensity with two bands

Table 2. Results of acid phosphatase electrophoretic phenotypes of *Ascochyto rabiei* isolates used in this study.

Electrophoretic phenotypes	Isolates	Locations	Phenotype frequency
EP-1	P-1	FatehJang	0.13
EP-1	P-4	Tarnab	---
EP-2	P-11	Chakwal	0.06
EP-3	P-13	Chakwal	0.13
EP-3	P-39-R	Kullorkot	---
EP-4	P-23	Attock	0.2
EP-4	P-CH	Chakwal	---
EP-4	P-KN-1	Kaghan	---
EP-5	P-26	Kaghan	0.26
EP-5	P-31	Kaghan	---
EP-5	P-2	Kaghan	---
EP-5	P-NC-1	Islamabad	---
EP-6	P-10	Chakwal	0.2
EP-6	P-FJ	FatehJang	---
EP-6	P-7	Tarnab	---

(very close to each other) at pH between 4.0-4.7 (EP-3). The highly aggressive, aggressive and moderately aggressive isolates were evenly distributed over the EP-1, EP-1 and EP-2 and EP-2 groups, respectively, whereas only one isolate P-4 belonging to EP-3 group, showed a unique pattern of double bands which was not found with any other isolate.

For further corroboration of banding patterns and different esterase activities, of the 7 isolates used number 4, 2 and 1 were respectively highly aggressive, aggressive and moderately aggressive. Assay were conducted with 2,3,4 and 6 days old mycelium for determination of enzyme activity with para nitrogphenyl acetate and enzyme activity with indoxyl acetate on the gels. The cellular protein concentration was higher in 3 and 4 days of mycelial growth. Esterase activity of 4 days old mycelium was found to range from 1.16-1.48 nkat, 1.45-2.60 nkat and 2.10 nkat/mg of protein, respectively, in highly aggressive, aggressive and moderately aggressive isolates. It was also observed that enzyme activity in cuvette was uniform or gradually increased in later mycelial growth in highly aggressive isolates whereas enzyme activity showed sudden decline in moderately aggressive isolates and mix observations were found in aggressive isolates.

When the growth of mycelium was prolonged, the intensity of the band remained uniform or gradually increased in case of highly aggressive as well as aggressive isolates whereas the intensity of the esterase band gradually declined in case of moderately aggressive isolates. On the basis of esterase activity determined with para nitrophenyl acetate, one can differentiate the highly aggressive, aggressive and moderately aggressive isolates whereas the banding pattern failed to support the established classification of aggressiveness.

Discussion

High degree of variability has been shown to exist in *Ascochyta rabiei* particularly in morphological appearance (Kaiser, 1973; Grewal, 1984), pathogenicity (Porta-puglia, 1992), phytotoxin production (Alam *et al.*, 1989, Hohl *et al.*, 1991) and at the DNA levels (Fischer *et al.*, 1995; Weising *et al.*, 1991). Genetic variability has recently been documented by DNA finger printing with synthetic oligonucleotides (Weising *et al.*, 1991) and by the PCR based RAPD method with arbitrary primers (Fischer *et al.*, 1995).

In the present studies, a certain degree of enzyme polymorphism was detected in the *A. rabiei* isolates. However, amongst the 7 enzymes studied, acid phosphatase and esterase showed polymorphism providing evidence of variability among isolates. There are reports of enzyme polymorphism in pathogenic fungi such as *Puccinia graminis* (Burdon & Roelfs, 1985), *Polyporus* spp., (Shannon *et al.*, 1973), *Fusarium oxysporum* (Bosland & Williams, 1987), *Cochliobolus carbonum* (Simcox *et al.*, 1992) and *Acremonium/Epichloe* spp. (Leuchtmann & Clay, 1990) at the level of the species/races/isolates.

The available collection of 40 isolates of *A. rabiei* represents different chickpea growing and partly separated regions of Pakistan like Attock, Chakwal, FatehJang, Islamabad, Kaghan, Kullorkot and Tarnab. These isolates when tested on 7 genotypes of chickpea for pathogenicity grouping showed differences in their pathogenicity as previously described by Weising *et al.*, (1991); Porta-puglia *et al.*, (1985); Hussain & Malik (1991). In the four groups developed on the basis of infection, none of the isolates was found in the less aggressive group. This result again documented that all the *A. rabiei* isolates collected in chickpea field show a considerable degree of pathogenicity. The 15 isolates used in this study were randomly selected so that they represent all 4 groupings as well as all locations in Pakistan (Table 1).

Where different growth periods of mycelium i.e., 2,3,4,6 and 8 days were used to measure the accumulation of cellular protein, maximum proteins were found in 3 and 4 days of mycelial growth irrespective of aggressiveness of isolates. Protein extracts of these periods were used because longer periods of growth led to protein extracts with lower concentrations and furthermore the number of high intensity bands decreased particularly in case of acid phosphatase.

The enzymes, Fumarase (FUM), alcohol dehydrogenase (ADH), lactate dehydrogenase (LDH), were found to be monomorphic whereas malate dehydrogenase (MDH) showed inconsistent banding that was difficult to resolve because of streaking or smearing, while glutamate dehydrogenase (GuDH) did not produce any bands on the gel. Similar results have been reported by Simcox *et al.*, (1992), who described that LDH, FUM and GuDH were monomorphic or showed less desirable degree of staining intensity or uniformity when races of *C. carbonum* were tested for these enzymes. Similarly, studies on MDH in *P. graminis* (Burdon & Roelf, 1985; Burdon *et al.*, 1981) provided the same results as shown for *A. rabiei*.

Two enzymes, acid phosphatase and esterase showed polymorphism. In case of acid phosphatase, we found variation in banding patterns in *A. rabiei* isolates (Fig.1). There are several reports that acid phosphatase is monomorphic with single band in

Acremonium/Epichloe spp., (Leuchtmann & Clay, 1990) and or two bands in *F. oxysporum* (Bosland & Williams, 1987) and polymorphic with 2 to 4 anodic bands in *Polyporus* spp., (Shannon *et al.*, 1973). Similarly, esterase activity with indoxyl acetate showed polymorphism having simple pattern of single or double bands with low and high intensity as compared with acid phosphatase. In several reports esterase activity on the gel is monomorphic in *P. graminis tritici, F. oxysporum* and *Pyricularia oryzae* and polymorphic in *P. recondita tritici, Polyporus* spp., and in *C. carbonum* (Simcox *et al.*, 1992; Bosland & Williams, 1987; Leung & Williams, 1986; Burdon & Roelf, 1985; Burdon *et al.*, 1981; Shannon *et al.*, 1973).

The isolates used in these studies were few and do not represent the whole population of the fungus for its virulence. Whereas genitical diversity is reported by Weising *et al.*, (1991) and Fischer *et al.*, (1995) in *Ascochyta rabiei* since no clear cut relationship was obtained in isozyme and virulence of the fungi that differentiate the aggressive isolates from other groups. There is need to analyse more isolates of different virulence as well as of different enzyme system.

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