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Variation in the Aggressiveness of *Phytophthora infestans* Pathotypes Collected from Different Potato Fields of Khyber Pakhtunkhwa (Pakistan)

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Abstract

Ten single lesion isolates of *Phytophthora infestans* collected from naturally late blight infected fields of different regions of the Khyber Pakhtunkhwa province during 2011 were characterized for aggressiveness based on their infection frequency, latent infection period, lesion area, lesion expansion rate and relative area under lesion expansion curve after their inoculation onto detached leaflets of potato cultivar Desiree. Significant variations were observed among different isolates originating from different locations for their aggressiveness potential and epidemiological components. Aggressiveness level determined on composite aggressiveness indices was also variable for the studied isolates. The study indicated that population of *P. infestans* in the Khyber Pakhtunkhwa province comprises diverse isolates with low to high aggressiveness potentials as measured by the epidemiological components. © 2014 Friends Science Publishers

Keywords: Aggressive strains; Epidemiology; Late blight; Mating type; *mt*DNA-haplotype

Introduction

Phytophthora infestans (Mont.) de Bary is a notorious plant pathogen responsible for the recurrence of huge yield losses of potato (*Solanum tuberosum* L.) and tomato (*Lycopersicon esculentum* L.) crop grown in different parts of the world (Fry *et al*., 2009). Since its first devastating role in the Irish potato famine in 1845, the pathogen has been found to have caused many epidemics of late blight disease and it still remains one of the challenging problems in global agriculture causing significant crop and monetary losses (more than 3 billion dollars annually) to the potato growers (CIP, 1996; Fry, 2008). In recent years, new genotypes and more aggressive strains of the pathogen has been reported from around the world, with high genetic diversity, aggressive behaviors and resistance to certain systemic fungicides commonly used for control of late blight disease (Spielman *et al*., 1991; Fry *et al*., 1992; Goodwin, 1997; Carlisle *et al*., 2001). It is believed that before the 1970s, global population of *Phytophthora infestans* in most part of the world (outside of Mexico) consisted of A1 mating types the asexually reproducing clones and relatively of low genetic diversity (Fry *et al*., 1992; Samen *et al*., 2003). A2 mating types were first reported from Switzerland in 1981 (Hohl and Iselin, 1984) and subsequently from Europe, America and other parts of the world (Fry *et al*., 1992; Goodwin *et al*., 1998), changing the old population of *P. infestans* with genetically diverse and aggressive new population comprising of both A1 and A2 strains with the ability to produce oospores through sexual reproduction, resulting in more difficult to control strategies implied for late blight management (Drenth *et al*., 1993; Day and Shattock, 1997; Fry, 2008; Flier *et al*., 2007). The displacement of old populations of the pathogen by new one is hypothesized to have occurred by several migrations of *P. infestans* from Mexico to other parts of the world possibly by shipment of seed tubers (Goodwin *et al*., 1994; Fry, 2008).

Aggressiveness, defined as the overall capability of pathogens to damage their hosts (Cooke and Deahl, 1998), is an important tool for detecting changes in the population structure of the pathogen of interest. The evidence that new populations of *P. infestans* are more aggressive than the older ones has been variably documented in the literature. Miller *et al*. (1998) reported differences in population of *P. infestans* of the US based on investigating the aggressiveness components of 22 isolates collected from Oregan and Washington on detached and whole plant experiments. Variations between isolates collected from the Northern Ireland for latent period, sporulation capacity, infection frequency and area under lesion expansion curve were documented (Carlisle *et al*., 2002). Similarly, variation in the aggressiveness parameters among isolates of *P. infestans* from Ecuador (Chacon *et al*., 2007), Belarus (Pliakhnevich and Ivaniuk, 2008) and South America

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(Andrade-Piedra *et al*., 2005) have been previously published.

In Pakistan, the occurrence of *P. infestans* was first documented from Swat Valley in 1984 (Khan *et al*., 1985) followed by scientific investigations, which were mainly focused on fungicides application targeting the pathogen to minimize yield losses of potato. Some years later, Ahmad and Mirza (1995) reported the presence of A2 mating type in Pakistani population of *P. infestans*. Since then some workers have studied mating types and resistance to metalaxyl fungicides of the pathogen (Batool *et al*., 1998; Ahmad, 2000; Ahmad *et al*., 2008), however, studies on aggressiveness of *P. infestans* in detached leaf assays and field experiments have not been carried out so far to our knowledge.

Although changes in population structure of *P. infestans* are generally monitored on the basis of mating types, mtDNA-haplotype, Glucose-6-phosphate isomerase (Gpi), RFLP and peptidase (Pep) analysis and SSR markers (Cooke and Lees, 2004; Lees *et al*., 2006; Pule *et al*., 2013), however, such techniques are either unavailable in developing countries like Pakistan or very expensive. Thus, characterization of population of *P. infestan* through simple aggressiveness parameters might be helpful in detecting quick and short term population changes of this pathogen. This study was conducted with the aim of studying variation in the aggressiveness of *P. infestans* occurring naturally in different areas of the Khyber Pakhtunkhwa province of Pakistan.

Materials and Methods

Collection of Plant Material

Potato fields of different areas of the Khyber Pakhtunkhwa province were visited in potato growing season during 2011. At least thirty samples of naturally late blight infected leaves from each field were collected from different potato fields of Khyber Pakhtunkhwa (Table 1), kept in dark polythene bags and transferred to Plant Pathology Laboratory, Arid Agriculture University, Rawalpindi for culturing *P. infestans* isolates. Ten isolates representing each locality were tested for aggressiveness components.

Culturing of *Phytophthora infestans* Isolates

Isolates of *P. infestans* were cultured using rye agar medium at Plant and Environmental Protection unit, National Agriculture Research Centre (NARC), Islamabad in August, 2011. Infected tissues of collected leaves (single lesion) from each locality were placed in petri-dishes containing rye agar medium amended with antibiotic 100 µL/mL vancomycin, 100 µL/mL pimaricin and 50 µL/mL rifamycin (Caten and Jinks, 1968). For initiation of sporangia formation, petri dishes were incubated for 4 days at 18ºC in dark. Sporangia were transferred by sterilized glass rod to fresh rye agar medium without antibiotics and were re-incubated at 18ºC for 14 days in dark. Newly formed sporangia were then disentangled by sterile glass rod and adding 10 mL of distilled water to each petri dish. Sporangial suspensions were filtered through a double layer of cheese cloth for removing mycelia fragments and concentrations were fixed by haemocyto-meter to 60000 sporangia/mL. The sporangia were refrigerated at 4ºC for two h to release zoospores and for use of inoculation (Pliakhnevich and Ivaniuk, 2008).

Table 1: Sample collection and details of visited locations of KPK

Locations	Fields visited	Samples collected	Isolates cultured	Isolates obtained	Abbreviation assigned to isolates
Kaghan	5	80	40	18	Ka
Naran	3	50	30	12	Nr
Sharan	4	54	20	14	Sh
Shougran	6	60	20	17	Sg
Batakundi	3	40	20	14	Bt
Balakot	4	40	30	12	Bl
Ayyubia	2	30	20	11	Ab
Bara Gali	3	55	40	14	Bg
Mahaban	2	30	20	11	Mb
Shabqadar	9	40	20	16	Sb

Detached Leaf Assay, Inoculation and Experimental Design

Leaves of uniform size were detached from eight weeks old potato plants (cv. Desiree) and thoroughly washed with distilled water, dried with clean bolting paper. Five leaflets were placed adaxial side up in each petri dish containing moist filter paper. Each isolate was inoculated onto five leaflets as a single drop of 20 µL zoospores suspension transferred to the midrib of each leaflet in the center on adaxial surface. The experiment was repeated three times. Each time experimental layout was completely randomized design (CRD) manner, considering each petri dish a single experimental unit further replicated four times. Petri dishes were incubated at 18ºC in an incubator with 12 h photoperiod for nine days.

Aggressiveness Components

Post inoculation observations on detached leaflets were made every 24 h for nine days. The aggressiveness components as previously documented (Spielman *et al*., 1992; Miller *et al*., 1998; Lebreton *et al*., 1999; Carlisle *et al*., 2002; Flier *et al*., 2003) were determined for detached leaflet assay as (a) percent infection frequency (IF), measured after ninth day of inoculation as no infection on leaf = 0%, infection on one leaf = 20%, three leaves infected = 60%, four leaves infected = 80% and five leaves infected = 100% following the method of Carlisle *et al*. (2002). (b) Latent infection period (LIP) (days), calculated as time

taken (days) from infection till the development of sporangia on the infection site (Kato *et al*., 1997; Chacon *et al*., 2007). For calculating LIP, five well-developed lesions from detached leaflet were randomly selected from the replicates and observed with naked eyes from the day of inoculation till the appearance of sporangia. (c) Lesion size was calculated as lesion area (LA) (mm^2) by measuring length and width of each lesion in both experiments. Observations were made with the naked eye every 24 h after inoculation. Lengths and widths were measured using a calibrated ruler (Chacon *et al*., 2007). Lesion area was used to estimate lesion expansion rate (LER) (mm^2/day) as previously described (Colon *et al*., 1995; Vleeshouwers *et al*., 1999). (d) Relative area under the lesion expansion curve (RAULEC) was measured according to Carlisle *et al*. (2002).

To determine aggressiveness level (%) of isolates, composite aggressiveness index (CAI) was calculated by CAI = (IF x LA)/LIP following the method of Montarry *et al*. (2007). Isolates with CAI ≥ 1000 were considered as highly aggressive; CAI ≥ 500 < 1000 as moderately aggressive and CAI ≤ 500 as weakly aggressive.

Data Analysis

In order to reduce the biasedness of results, the experiment was repeated three times, each time further replicated four times. Data was averaged across the three experiments and used for data analysis. Multivariate analysis of variance (MANOVA) was performed using SPSS software v. 19 (IBM Corp, 2012) for measuring variation among the studied isolates for the aggressiveness parameters. Significant differences among the variables means were evaluated by Least Significant Difference (LSD) at $p \leq 0.05$.

Results

Our results demonstrated significant variations in the aggressiveness potentials, measured as epidemiological components, of the isolates of *P. infestans* collected from different fields of the Khyber Pakhtunkhwa province (Table 2). Out of ten regions, only isolates collected from Shabqadar (Sb) area were found to be weakly aggressive. All other isolates were either moderately or highly aggressive. Based on the aggressiveness tests, our study indicated that population of *P. infestans* in the studied regions of Khyber Pakhtunkhwa consisted of diverse isolates in regard to their aggressiveness. Out of ten regions, 10% isolates were weakly aggressive (CAI ≤ 500), 50% moderately aggressive (CAI ≥ 500 < 1000) and 40% highly aggressive with composite aggressiveness index greater than 1000 (Table 3).

Infection Frequency (IF)

Differences among infection frequencies of isolates in each group classified on the bases of their aggressiveness in Table 3 were non-significant however, significant ($p \leq 0.05$) among the groups in the order highly aggressive > moderately aggressive > weakly aggressive (Fig. 1A). Isolates collected from Sharan, Batakundi, Ayubia and Bara Gali (Sh, Bt, Ab, Bg) were highly aggressive (Table 3) and hence resulted in the highest infection frequencies i.e., 95.6, 96.06, 96.11 and 96.19% respectively, on detached leaves. Likewise, moderately aggressive isolates Ka, Nr, Sg,

Table 2: Multivariate analysis of variance (MANOVA) of different isolates of *Phytophthora infestans*

Source	Aggressiveness components	Type III Sum of Squares	d.f.	Mean Square	F	Sig.	Observed Power[b]
Corrected Model	IF	4584.695[a]	9	509.411	665.604	.000	1.000
	LIP	56.697[c]	9	6.300	511.479	.000	1.000
	LA	3486.077[d]	9	387.342	1772.316	.000	1.000
	LER	16.417[e]	9	1.824	41.785	.000	1.000
	RAULEC	2021.657[f]	9	224.629	1342.649	.000	1.000
Isolates	IF	4584.695	9	509.411	665.604	.000	1.000
	LIP	56.697	9	6.300	511.479	.000	1.000
	LA	3486.077	9	387.342	1772.316	.000	1.000
	LER	16.417	9	1.824	41.785	.000	1.000
	RAULEC	2021.657	9	224.629	1342.649	.000	1.000
Error	IF	22.960	30	.765			
	LIP	.369	30	.012			
	LA	6.557	30	.219			
	LER	1.310	30	.044			
	RAULEC	5.019	30	.167			
Total	IF	285262.288	40				
	LIP	2186.332	40				
	LA	283532.609	40				
	LER	378.387	40				
	RAULEC	16457.938	40				

[a]R Squared = .999 (Adjusted R Squared = .999); [c]Computed using alpha = .05; [c]R Squared = .956 (Adjusted R Squared = .943); [d]R Squared = .999 (Adjusted R Squared = .998); [e]R Squared = .946 (Adjusted R Squared = .931); [f]R Squared = .992 (Adjusted R Squared = .989)

IF: Infection frequency; LIP: , latent infection period; LA, lesion area; LER, lesion expansion rate; RAULEC, relative area under lesion expansion curve

Bl and Mb showed 78.907, 78.735, 76.485, 77.815 and 77.063% IF. Lowest infection frequency 64.65% was observed for isolates collected from Shabqadar (Sb).

Latent Infection Period (LIP)

Latent infection period calculated in days, were significantly higher (9.58 days) for the weakly aggressive isolate Sb, followed by moderately aggressive isolates Ka, Nr, Sg, Bl and Mb (7.74-8.015 days) and highly aggressive isolates Sh, Bt, Ab, Bg which corresponded to significantly lowered LIPs ranging from 5.925 to 5.995 days respectively (Fig. 1B). Differences among latent infection periods were insignificant among isolates of the same group i.e., highly aggressive, moderately aggressive or weakly aggressive.

Lesion Area (LA)

Isolates collected from different geographic regions of Khyber Pakhtunkhwa showed differential effects on lesion area development. Lesion areas (mm^2) calculated for different isolates were compared with each other for differences. Isolates of highly aggressive class were found to have maximum impact on lesion area development and values regarding LA for this class ranged from 92.38 to 94.227 (Fig. 1C). Similar but comparatively less aggressive behavior was exhibited by isolates Ka, Nr, Sg, Bl and Mb corresponding to LAs as 79.105, 79.493, 77.767, 80.65 and 82.623 mm^2 respectively. Significantly lowered value of LA 63.48 mm^2 was calculated for isolates collected from Shabqadar (Sb). Contrarily to other epidemiological components, differences among values of lesion area were significant ($p \leq 0.05$) for almost all isolates (Fig. 1C).

Lesion expansion rate (LER)

Significant differences among isolates were observed for LER with lowest value 1.48 mm day^{-1} recorded for isolates collected from Shabqadar (Sb) and highest value 4.047 mm $day^{-1}$for isolates originating from Sharan (Sh)(Fig. 1D). Isolates obtained from Kaghan (Ka), Naran (Nr), Balakot (Bl), Ayyubia (Ab) and Mahaban (Mb) had almost similar LER values (2.82-3.045 mm day^{-1}) with slight differences. Results revealed that isolates collected from Batakundi (Bt) and Bara Gali (Bg) contributed to 3.605 and 3.41 mm day^{-1} lesion expansion rate.

Relative Area under Lesion Expansion Curve (RAULEC)

Variations for relative areas under lesion expansion curve were significant among all isolates. Regarding to differences in RAULEC values, the least aggressive isolates originating from Shabqadar (Sb) caused the lowest RAULEC value 7.54 while highest values (31.065) were recorded for isolates originating from Bara Gali (Fig. 1E).

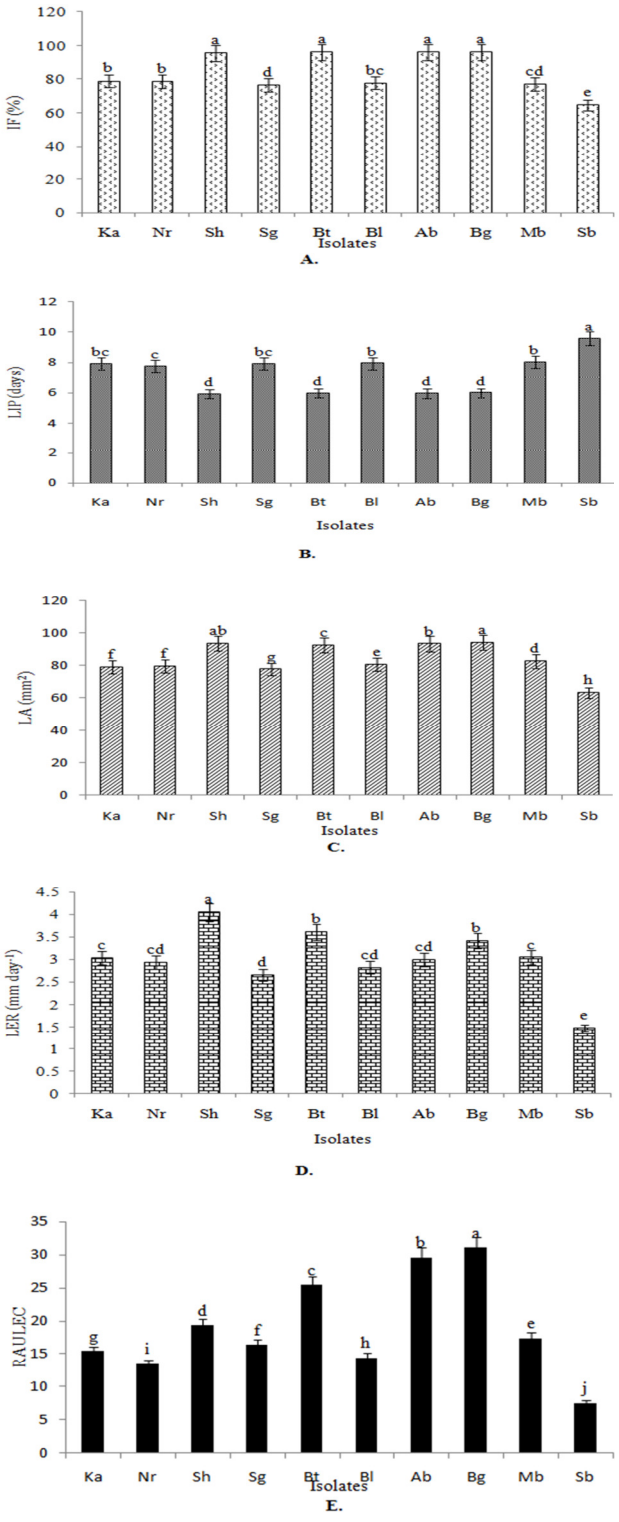

Fig. 1(A-E). Variations among different isolates of P. infestans for the aggressiveness components; A-Infection frequency, B-Latent infection period, C-Lesion area, D-Lesion expansion rate, E-Relative area under lesion expansion rate

Unlike other aggressiveness parameters, almost all isolates of *P. infestans* corresponded to differential values of RAULEC, each isolate differing significantly from other. The order of isolates which revealed different RAULECs (highest to lowest values) were Bg > Ab > Bt > Sh > Mb > Sg > Ka > Bl > Nr > Sb.

Discussion

Aggressiveness is the quantitative measurement of disease damage caused by pathotypes on susceptible hosts (Plank, 1963). In practice, different epidemiological components like infection efficiency, latent period, incubation period, sporulation capacity, lesion size, lesion growth rate, area under lesion expansion curve and maximal curve growth rate are selected by different plant pathologists dealing with *P. infestans* for the assessment of aggressiveness (Miller *et al*., 1998; Flier and Turkensteen, 1999; Pliakhnevich and Ivaniuk, 2008; Pariaud *et al*., 2009). Selection of components for aggressiveness measurement is usually based on geographic regions, experiment i.e., wither detached leaflet or whole plant assay and the objectives of the study. Several authors have revealed the reliable efficacy of one or more of these components for detecting changes in global population structure of *P. infestans* and attribute the replacement of indigenous old population by new population worldwide to the increased pathogenicity and aggressiveness of the new population of *P. infestans* (Pliakhnevich and Ivaniuk, 2008; Fry *et al*., 2009). IF, LP, LA, LER and RAULEC are important epidemic parameters denoting the pathogen's capacity to cause damage on the host. Based on variation in these components, different genotypes of the pathogen under study may be considered as more aggressive or less aggressive. In addition to aggressiveness components, modern techniques like mtDNA-haplotype, Glucose-6-phosphate isomerase (Gpi), RFLP and peptidase (Pep) analysis and SSR markers may further augment the detection of aggressive strains and population changes of *P. infestans* in in different geographic regions (Cooke and Lees, 2004; Lees *et al*., 2006; Pule *et al*., 2013).

Our aim was to detect variability in isolates collected from different regions of Khyber Pakhtunkhwa on detached leaflet assay based on epidemiological parameters which detected significant variations among the tested isolates. Based on composite aggressiveness indices, isolates were grouped into three classes i.e., highly aggressive, moderately aggressive and weakly aggressive (Table 3). This grouping was not confined to only CAI, in fact, almost all aggressiveness parameters varied accordingly with aggressiveness level of the isolates, although slight deviations were observed in some cases (Fig. 1A-5). IF, LIP, LA, LER, RAULEC and CAI were lowest for isolates collected from Shabqadar (Sb), suggesting that population of *P. infestans* in Shabqadar consists of weakly aggressive isolates. Conversely, it is clearly demonstrated that isolates representing other potato growing regions in this study were either highly or moderately aggressive. The variability among isolates for overall aggressiveness components have also been reported in Ecuador (Chacon *et al*., 2007), Europe (Carlisle *et al*., 2002; Lehtinen *et al*., 2009), Belarus (Pliakhnevich and Ivaniuk, 2008), USA (Miller *et al*., 1998) and South America (Andrade-Piedra *et al*., 2005).

Table 3: Aggressiveness level and composite aggressiveness indices of *Phytophthora infestans*' isolates

Aggressiveness level of isolates	Isolates	Composite aggressiveness index (CAI)	Percentage of isolates based on aggressiveness
	Group A		
Highly Aggressive	Sh	1511.28a	40 %
	Bt	1482.32a	
	Ab	1505.90a	
	Bg	1512.00a	
	Group B		
Moderately aggressive	Ka	789.11b	50 %
	Nr	991.36b	
	Sg	751.67b	
	Bl	790.89b	
	Mb	794.40b	
	Group C		
Weakly aggressive	Sb	428.39c	10 %

Since first report of the occurrence of A2 mating type in Switzerland in 1981 (Hohl and Iselin, 1984), changes in population structure of *P. infestans*, replacing the relatively less virulent 'old' by more aggressive 'new' populations in American, European and other countries of the world have been frequently documented (Drenth *et al*., 1993; Day and Shattock, 1997; Fry, 2008; Flier *et al*., 2003). Although in Pakistan, some authors have reported changes in population of *P. infestans* since 1995, however, their studies were mainly based on A2 mating types and metalyxyl sensitivity and did not address aggressiveness components of the pathogen (Ahmad and Mirza, 1995; Batool *et al*., 1998; Ahmad, 2000; Ahmad *et al*., 2008). This study highlights that population structure of *P. infestans* in the Khyber Pakhtunkhwa province has been replaced by more aggressive strains (40% highly aggressive, 50% moderately aggressive and only 10% weakly aggressive) replacing the old relatively less aggressive strains; hence further studies are needed to study population dynamics of the pathogen sampling isolates from all parts of the country where late blight occur.

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