Pak. J. Bot., 53(5): 1875-1882, 2021. DOI: http://dx.doi.org/10.30848/PJB2021-5(44)

DISTRIBUTION PATTERN OF SOUTHERN CORN LEAF BLIGHT IN KHYBER PAKHTUNKHWA-PAKISTAN AND ITS PCR BASED DETECTION IN ASYMPTOMATIC LEAVES AND PLANT DEBRIS

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Abstract

Southern corn leaf blight, caused by *Bipolaris maydis,* is an important disease of maize in Khyber Pakhtunkhwa (KP) province, Pakistan. Extensive surveys were carried out during kharif 2015 to assess the distribution pattern of the disease in various districts of KP. The disease was prevalent in all surveyed areas with varying degree of severity. Plains of KP were more prone to the disease due to favorable environmental conditions than highlands. A direct PCR protocol for detection of the pathogen in asymptomatic leaves was developed using species specific primers JB588/JB591, JB587/JB596 and JB588/JB598. Primer pairs JB588/JB591 and JB587/JB596 were equally effective in detecting the pathogen as compared to the primer pair JB588/JB598. Nested PCR was however required to detect the pathogen in plant debris. Primer pair JB587/JB596 proved reliable in detecting the pathogen in plant debris through nested PCR.

Key words: Southern corn leaf blight, *Bipolaris maydis,* Khyber Pakhtunkhwa (KP), PCR based detection, Nested PCR.

Introduction

Southern corn leaf blight (SCLB) caused by *Bipolars maydis* (Nisikado and Miyake) Shoem, is a serious disease of maize worldwide. The disease flourishes well in warm and humid environment within a temperature range of 20-32^{o}C (Shah *et al.,* 2007; Martinez *et al.,* 2010). Such environmental conditions frequently prevail in the plains of Khyber Pakhtunkhwa (KP) province Pakistan during Kharif season (Razzaq *et al.,* 2019). Therefore the prevalence of the disease in central zone of the province is no surprise (Ali *et al.,* 2011). However, no attempt has been made in the past to document severity and distribution pattern of the disease across KP. Such data arguably will prove useful in disease mapping and adoption of appropriate management strategies such as deployment of resistance against the disease. Such information could reveal disease foci in the area that prove useful for studying epidemiology of the disease.

The polycyclic nature of the disease and saprophytic ability of the pathogen makes it difficult to control under favorable environmental conditions (Blanco & Nelson, 1972). Therefore, a precise and rapid detection of the pathogen in plant debris and asymptomatic plant tissue prior to the appearance of symptoms is necessary for adoption of timely disease management strategies. Conventional detection involves isolation, culturing and comparing the cultural characteristics with those in the published keys. However, such methods are time consuming and require expertise (Grote *et al.,* 2002; Capote *et al*., 2012). Currently, PCR based methods have improved the detection of pathogens (Schaad *et al.,* 2007). These techniques have an advantage over conventional detection methods as pathogen does not need to be cultured and PCR can detect even a single nucleotide molecule in a complex mixture (Mirmajlessi *et al.,* 2015). Specific oligonucleotide primers based on a certain nucleic acid sequences act as diagnostics for the pathogen and utilized by the PCR. Qing-zhou (2017) designed species specific primers Y-EF-F and Y-EF-R for the detection of *Bipolaris zeicola* in artificially inoculated plants. Beck (1998) identified primers from the internal Transcribed Spacer (ITS) DNA sequences of ribosomal RNA gene of *B. maydis.* On the other hand, Kang *et al.,* (2018) designed primers from polyketide synthetase gene (PKS) of *Cochliobolus carbonum* and non-ribosomal peptide synthetase gene (NRPS) of *C. heterostrophus* for detection in infected seeds through duplex PCR.

Conventional PCR sometimes fails to detect the pathogen in situation where the concentration of the pathogen DNA is either low or PCR inhibitors are present in template DNA (Yang *et al.*, 2017). This can be overcome by the use of a more advanced PCR technique such as nested PCR which increases sensitivity and specificity (Olmos *et al.*, 1997; Josefsen and Christiansen, 2002). Nested PCR is a two round PCR where two primer pairs are used successively to amplify the target DNA (Haqqi *et al*., 1988). During the first round, a large amplicon of pathogen DNA is amplified with an external primer pairs. PCR products from the first round are then diluted and re-amplified by an internal primer pair (Porter-Jordan *et al.*, 1990; Cai *et al.*, 2014). Studies on detection of different plant pathogen using nested PCR have been reported previously (Mercado-Blanco *et al.*, 2001; Ippolito *et al*., 2002; Bhat & Browne, 2010; Qin *et al*., 2011; Yang *et al.*, 2017). Hou *et al.*, (2013) developed a semi nested PCR protocol for detection of *Curvularia lunata.* Primer pair ClgD1/ClgD3 in the first round and ClgD2/ClgD3 in second round of nested PCR was used to detect the pathogen in infected plant leaves.

The aim of the present study was to determine the distribution pattern of southern corn leaf blight in Khyber Pakhtunkhwa province and to develop a reliable PCR protocol for early detection of the pathogen in asymptomatic tissues and plant debris.

Materials and Methods

Sampling procedure and study area: Various districts of Khyber Pakhtunkhwa (KP) province, including Peshawar, Nowshera, Swabi, Charsadda, Mardan, Kohat and Haripur were surveyed during Kharif season 2015, at flowering stage of the maize crop to determine the distribution pattern of SCLB based on disease severity data. Eight sites per district were selected and five fields per site at a distance of 2 km apart were surveyed. The location of each field was marked with Global Positioning System (GPS). The methodology comprised three stage cluster sampling (multistage), as sites are nested within districts and fields are nested within sites.

Assessment of southern corn leaf blight: Data on disease severity in each field was assessed at five random points and average of these was considered as one reading. For sampling, $1m^2$quadrate was used. The quadrate was thrown randomly in the field and data on disease severity was recorded on 4-5 randomly selected plants within the quadrate, using the rating scale of 0 – 5 (Sharma, 1983). According to this scale 0; represents no disease, 1; refers to few lesions on lower leaves. 2; shows moderate lesions on lower leaves only, 3; exhibits increased amount of lesion on lower and moderate on middle leaves, 4; abundant lesion on lower and middle leaves exceeding to the upper leaves, 5; all leaves are severely infected and plant is nearly dead. For further accuracy in disease severity documentation an arbitrary graduation of 10 classes 0.5, 1.0. 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5 and 5.0 were made.

Nested design was used for the analysis of disease severity data, Social statistical package IBM SPSS 20 was used for the analysis. Disease maps were constructed using Arcview GIS software for determination of spatial pattern of the disease.

Detection of *Bipolaris maydis* in asymptomatic plant tissue and plant debris

Collection of samples: Leaves without any noticeable disease symptoms were collected from six different fields, before the onset of the disease. The samples were designated as Asyptomatic Plant leaves (APL) in a series APL1 to APL6.

Plant debris of a previously infected crop was also collected from seven different fields, one month after the harvest of the crop. The samples were designated as Plant Debris (PD) in a series and labeled as PD1 to PD7.

DNA extraction from plant tissues and debris: Asymptomatic plant leaves and debris of the previous crop were surface disinfested with 70 % ethanol and washed with double distilled water. DNA was extracted using CTAB method (Murray and Thompson, 1980) with some modifications.

Plant material (200mg) was uniformly homogenized in sterilized pre chilled mortar and pestle. Further homogenization of the samples was achieved in liquid nitrogen with continuous crushing. Each sample was suspended in 600ul CTAB buffer in 1.5 ml Eppendorf tube. The samples were then incubated in water bath at 65°C for 30 minutes with continuous gentle shaking. The suspension was de-proteinized with 700ul phenol chloroform isoamyl alcohol (25:24:1) and vortexed briefly followed by centrifugation at 12000 rpm for 12 min. The supernatant was transferred to another tube to which ammonium acetate (28ul) and ice chilled absolute ethanol was added and kept at -20°C for 1 hour or overnight. Further centrifugation was performed at 10000 rpm for 15 min. The supernatant was discarded and pellet was washed with ice chilled 350ul of 70% ethanol, followed by centrifugation at 12000 rpm for 5min. Finally, the DNA pellet was suspended in 40ul TE buffer.

Detection of *Bipolaris maydis* in plant tissues: The extracted DNA from asymptomatic plant tissues was tested for the presence of *Bipolaris maydis*, using Polymerase Chain Reaction (PCR). Pairs of species specific primers, designed from the Internal Transcribed Spacer (ITS) DNA sequences of the ribosomal RNA gene of the pathogen (Beck, 1998), were used during the study ((Table 1). The procedure is briefly described below.

Six samples of the target DNA of the pathogen in the asymptomatic plant tissue along with positive and negative control were amplified using PCR. An aliquot of 20ul master mix (including, 10ul Dream Taq green master mix by Thermo scientific, 2ul extracted genomic DNA, 1ul each of forward and reverse primer and 6ul PCR water) was prepared for each PCR reaction. The positive control consisted of previously amplified genomic DNA of *B. maydis* in PCR master mix. In negative control, DNA was replaced by sterilized distilled water in the master mix. Temperature profile having 94°C, initial denaturation for 3 min, followed by 40 cycles of denaturation at 94°C for 30 sec, annealing at 58°C for 30 sec, extension at 72°C for 1 min and final extension at 72°C for 5 min was used for PCR reaction. Electrophoresis in 2% (w/v) agarose gel having ethidium bromide using 1 x TBE buffer was achieved to separate the PCR amplification products. Visualization was performed in UV trans-illuminator and gels were subsequently photographed.

Detection of *Bipolaris maydis* in plant debris: Nested PCR was used to detect the pathogen in the genomic DNA extracted from plant debris using degenerate primers ITS1/ITS4 (Table 2) in the first round of nested PCR. Amplification was executed in PCR tube having 10ul Dream Taq green master mix (Thermo scientific), 2ul extracted genomic DNA, 1ul each of forward and reverse primer and 6ul HPLC (High Performance Liquid Chromatography) water. The thermal profile was optimized at 94°C initial denaturation for 3 min, followed by 40 cycles of denaturation at 94°C for 30 seconds, annealing at 58°C for 30 sec, extension at 72°C for 1 min and final extension at 72°C for 5 min. Second round of nested PCR was performed with species specific primers (Table 1) using the same constituent of master mix and temperature profile except that the genomic DNA was replaced by 1ul of first round PCR product. Positive and negative control was also included in the study.

Table 1. Pairs of species specific primers used for the detection of *Bipolaris maydis* (Beck, 1998).

Source	5 ' primer (5 '-3')	3' primer(5 '-3')	Product size (bp)
Bipolaris maydis	JB 588 CACCCATGTCTTTTGCGCAC	JB 591 CTCCTGATACAGAGTGCAAAA	413 bp
Bipolaris maydis	JB587 CAGTTGCAATCAGCGTCAGTA	JB596 'GAGGTCAAAAGTTAAAAATCGTAA	331 bp
Bipolaris maydis	JB 588 CACCCATGTCTTTTGCGCAC	JB598 CCGAGGTCAAAAGTTAAAAATCTA	465 bp

Table 2. Universal primers used for the amplification of DNA of *Bipolaris maydis*.

Primer	Sequence(5 '-3')	Product size (bp)	Annealing temperature (^{o}C)
ITS 1	TCCGTAGGTGAACCTGCGG	19	61.7
ITS 4	TCCTCCGCTTTATTGATATG	20	54.3

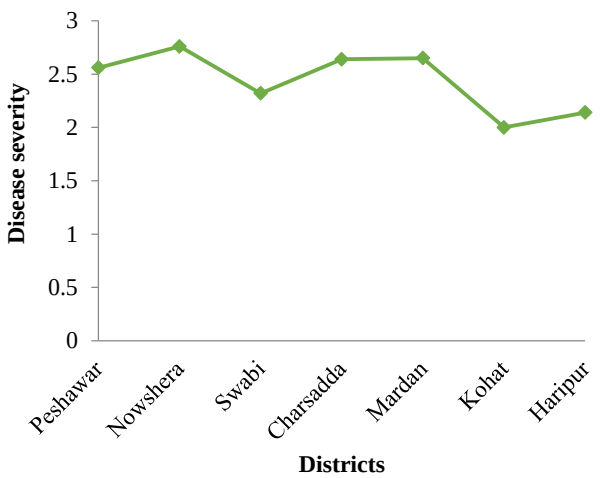

Fig. 1. Mean disease severity of southern corn leaf blight at various sites across Khyber Pakhtunkhwa.

Results

Severity of southern corn leaf blight in Khyber Pakhtunkhwa province: Southern corn leaf blight (SCLB) was prevalent in all fields visited during the survey. However its severity varied across the areas surveyed. Significant (p<0.01) interactive effect of districts and sites were evident in terms of disease severity. Disease severity ranged between 1.6 to 3.6 at all sites across districts (Table 3). The highest mean disease severity (3.6) was observed at site 4 (Gari Kapoora) of district Mardan, followed by site 8 (Pirsabak) and site 3 (Gujar Kas) of district Nowshera. Similarly, the lowest disease severity (1.6) was reported from site 6 (Panian) of district Haripur, site 5 (Shah Pur) of district Kohat, site 2 (Shahbaz Gari) of district Mardan and site 6 (Shahmansoor) of district Swabi. Results also indicated that the disease was more severe in the low altitude areas of Nowshera, Charsadda, Mardan, Swabi and Peshawar as compared to the highlands of Haripur and Kohat (Fig. 1).

Distribution pattern of southern corn leaf blight in KP province: A disease severity map (Fig. 2) was constructed based on GPS (Global Positioning System) records and disease severity data collected from various sites in each districts of KP. Sites in the entire district were placed in four categories, with disease severity ranging from 1.6-2.0, 2.1-2.5, 2.6-2.9 and 3.0-3.6. Areas with disease severity range of 1.6-2.0 were termed as ″trace disease severity area″ (Pink), those with the range of 2.1-2.5 as ″low disease severity area″ (Blue) while areas exhibiting disease severity between 2.6-2.9 as ″moderate disease severity areas″ (Orange). Similarly, areas with a disease severity of 3.0-3.6 were marked as ″high disease severity area″ (Red).

Spatial pattern of SCLB epidemics with high disease severity (Red) was visualized at site 5 (Taru jabba) and site 8 (Warsak) of district Peshawar, site 3 (Gojar Kas), site 7 (Azakhel) and site 8 (Pirsabak) of district Nowshera, site 4 (Rajar), site 5 (Gulbela) and site 6 (Dargai) of district Charsadda, site 4 (Ghari kapoora), site 6 (Katlang), site 7 (Muqam) and site 8 (Gujar ghari) of district Mardan. No hot spot (Red) from Swabi, Kohat and Haripur was evident. Likewise, trace (Pink), low (Blue) and moderate (Orange) severities of the disease was reported from various sites of all districts visited during the study.

Detection of *Bipolaris maydis* in asymptomatic plant leaves: Species specific primer pairs of *B. maydis,* previously designed from the ITS region of ribosomal DNA were used to detect the pathogen in asymptomatic plant tissues. Primer pair JB588/JB591 amplified 413 bp fragments from sample APL1, APL2, APL4, APL5 while sample designated as APL3 and APL6 did not produce any band, indicating the absence of the pathogen in plant tissue. A band of the same size was also observed for positive control. Conversely, negative control did not give any amplification (Fig. 3A). Similar results were also observed for primer pairs JB587/JB596 (Fig 3B) and JB588/JB598 (Fig. 3C), where amplification was observed at 331 bp and 465 bp, respectively for most of the samples. However, the primer pair JB588/JB598 failed to produces any band for sample APL5 which was detectable with other primer pairs. The band for sample APL1 although present was comparatively weak.

Detection of *Bipolaris maydis* in plant debris: Direct PCR for the detection *B. maydis* in plant debris failed to give any visible bands. Therefore a comparatively more sensitive technique of nested PCR was adopted. In the first round of nested PCR using universal primers ITS1/ITS4, samples including PD2, PD3 and PD4 produced visible bands of 596 bp size. A band of the same size was also noticed for positive control.

During the second round of nested PCR, three primer pairs including JB588/JB591, JB587/JB596 and JB588/JB598 were used separately to detect the pathogen. Primer pair JB588/JB591 amplified 413 bp fragments from sample PD2, PD3, PD4 and PD6 (Fig 4A). Similarly, primer pair JB587/JB596 proved promising producing bright bands for all samples including positive control at 331bp (Fig. 4B). The third primer pair JB588/JB598 amplified bands of size 465 bp for all samples except PD4 (Fig. 4C).

Table 3. Disease severity of southern corn leaf blight at various sites in Khyber Pakhtunkhwa, Pakistan.

Districts	Area code	Sites	Altitude (m)	Disease severity	Mean Disease severity
Peshawar	1	Pandu	308	2.4	2.56
	2	Lala Kaly	295	2.4	
	3	Pushtakhara	293	2.7	
	4	Jhagra	299	2.7	
	5	Taru Jabba	299	3	
	6	Malakandher	357	2.2	
	7	Landy Akhun Ahmad	370	1.8	
	8	Warsak	340	3.3	
Nowshera	1	Kheshgi	300	1.9	2.76
	2	Meera	295	2.1	
	3	Gojar Kas	285	3.5	
	4	Pirpiye	291	2.9	
	5	Dheri Zardad	298	2.5	
	6	Rashakai	291	2.4	
	7	Azakhel	287	3.3	
	8	Pirsabak	288	3.5	
Swabi	1	Central Swabi	323	2.3	2.32
	2	Yar Hussain	315	2.3	
	3	Gohati	359	2.1	
	4	Sheikh Jana	350	2.6	
	5	Ismaila	314	2.8	
	6	Shahmansoor	307	1.6	
	7	Shawa adda	340	2	
	8	Maneeri	335	2.9	
Charsadda	1	Central Charsadda	291	2.1	2.64
	2	Sholgara	284	2.6	
	3	Sardheri	301	2.5	
	4	Rajar	300	3	
	5	Gul Bela	293	3.1	
	6	Dargai	301	3	
	7	Jogian	290	2.7	
	8	Nisata	283	2.1	
Mardan	1	Tawas kaly	299	2.1	2.65
	2	Shahbaz Gari	305	1.6	
	3	Bala Gari	303	2.1	
	4	Gari Kapoora	300	3.6	
	5	Tooro	287	2.4	
	6	Katlang	303	3	
	7	Muqam	285	3.2	
	8	Gujar Ghari	298	3.2	
Kohat	1	Nusrat Khel	576	2.1	2.0
	2	Muslim Abad	830	2.1	
	3	Mandoori	489	2.6	
	4	Laachi	458	2.1	
	5	Shah Pur	493	1.6	
	6	Bahadur Kot	726	1.7	
	7	Krapa	480	1.7	
	8	Jawzara	785	2.1	
Haripur	1	Mang	550	2.4	2.14
	2	Pind Kamal Khan	600	2.2	
	3	Meelam	810	1.8	
	4	Balter	549	2.3	
	5	Sarai Saleh	597	2.4	
	6	Panian	471	1.6	
	7	Khan Pur	520	1.8	
	8	Mankrai	610	2.6	

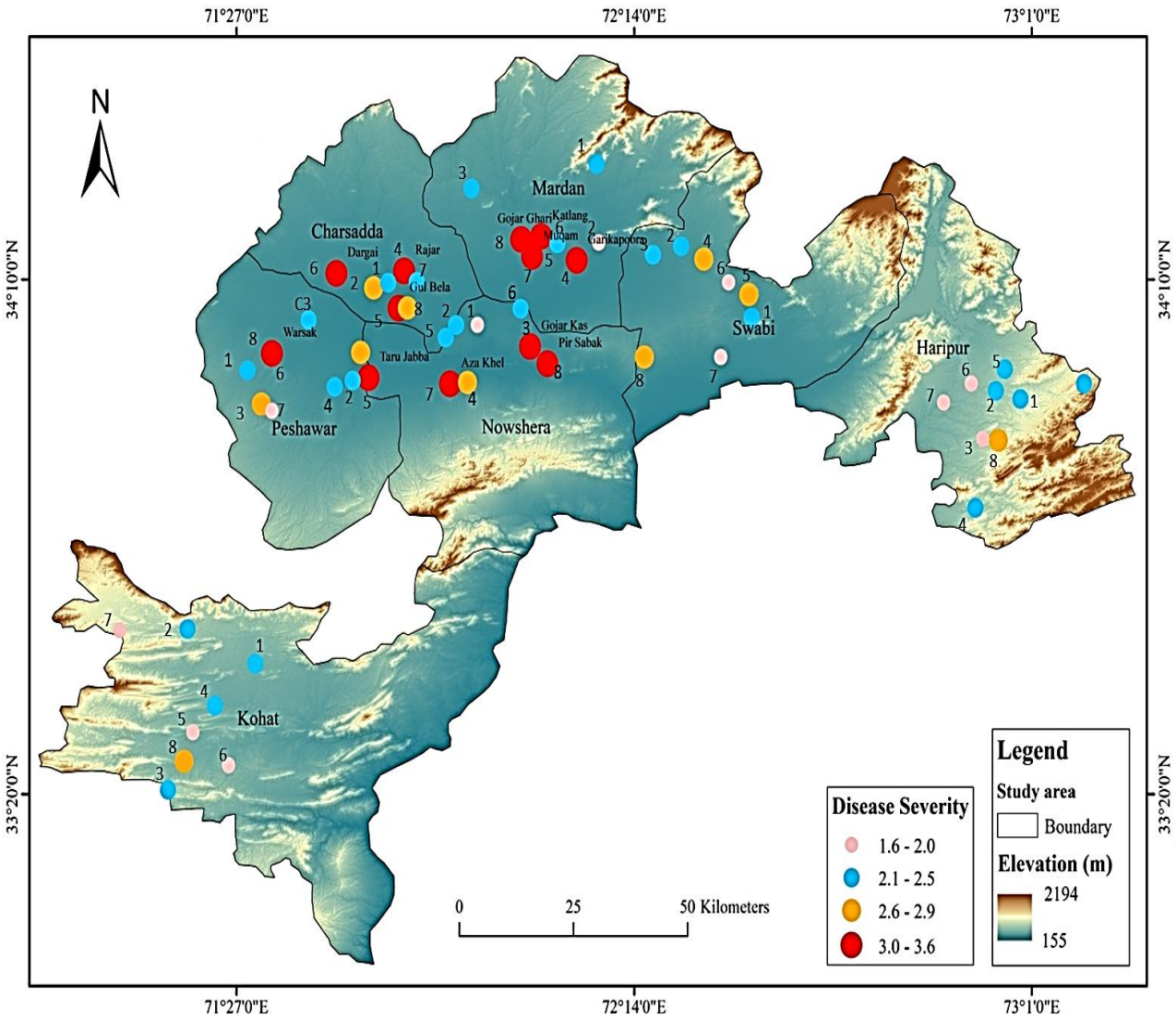

Fig. 2. Distribution pattern of southern corn leaf blight in Khyber Pakhtunkhwa province, Pakistan based on disease severity.

Discussion

Southern corn leaf blight (SCLB) is a major factor limiting maize production in Khyber Pakhtunkhwa (KP) province (Ali and Ahmad, 1992). Being polycyclic in nature, soon after introduction the disease becomes epidemic once the conditions become favorable. This warrants study of the distribution pattern of the disease at the earliest.

Major maize growing areas of KP including Peshawar, Nowshera, Swabi, Charsadda, Mardan, Haripur and Kohat were surveyed where it was found that the disease was present in all surveyed areas. However, its severity varied across the province. Previous studies have shown that the disease has a countrywide distribution (Hafiz, 1986) with epiphytotic severities reaching up to 75% (Maryum *et al*., 2011). In the present study, the disease severity were high in Nowshera, Charsadda, Mardan, Peshawar and Swabi district compared to Haripur and Kohat. This indicated that plains are more prone to the disease than highlands of KP. The results are in line with the previous findings where high prevalence and incidence of the disease have been reported from the areas of low altitude in various districts of Azad Jammu and Kashmir (Maryum *et al.*, 2011; Azam *et al.*, 2017) and Nepal (Paudel & Koirala, 1995).

Climatic conditions in central KP are warm and humid during the kharif season (Ali *et al.*, 2011) and more suitable for development of the disease. Hence, high disease severity recorded from central KP including plains of the province is not surprising. Lukens & Mullany (1972) observed more infestation in fields with greater morning shade and humid soil. Size and number of lesion, spore germination and appresorium formation on the crop is positively correlated with relative humidity (Rai *et al.*, 2002; Pei *et al.*, 2007). By contrast, southern areas including Kohat have warm and dry climate not suitable for disease development. Schenck & Shelter (1974) reported that long period of bright sunny days and dry conditions are not suitable for disease development. Moreover, fluctuating day and night temperature retards disease development than situation where day/night temperature does not fluctuate (Nelson & Tung, 1973). Furthermore, in northern areas of the province including Haripur, the climate remains cool and is therefore not conducive for disease development.

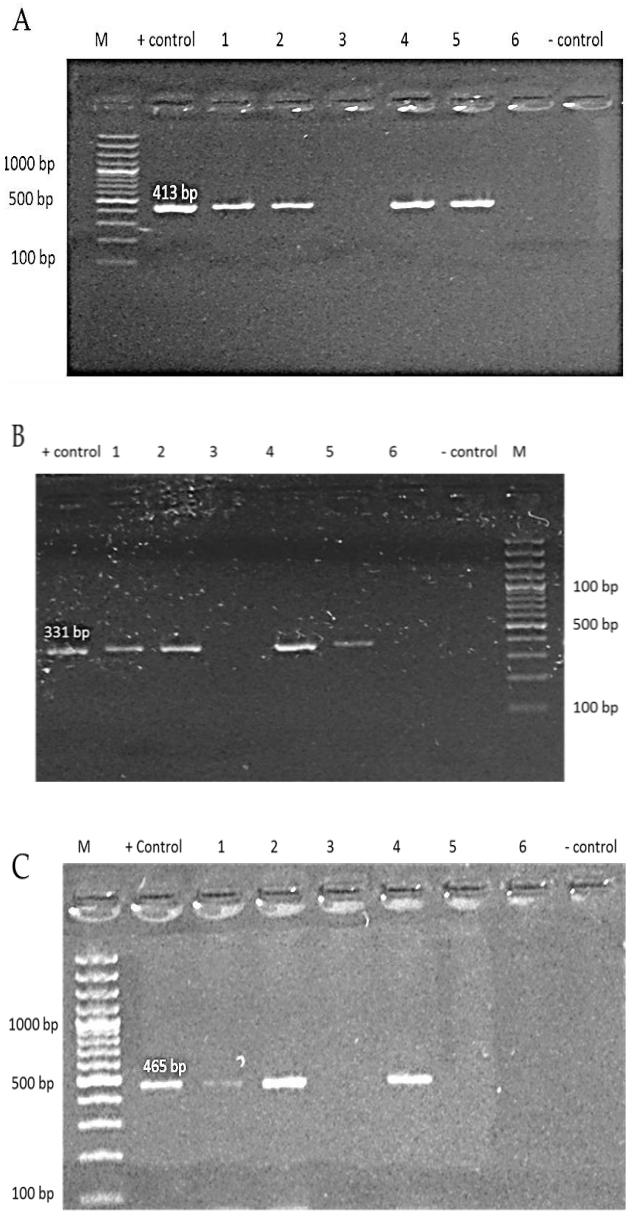

Fig. 3. Detection of *Bipolaris maydis* in asymptomatic plant tissue through direct PCR with primer pairs JB588/JB591(A), JB587/JB596(B) and JB588/JB598 (C) amplifying 413 bp, 331 bp and 465 bp bands respectively. Lane 1-6: DNA from Asymptomatic Plant Leaves (APL) labeled as APL1, APL2, APL3, APL4, APL5 and APL6, in PCR master mix, M: 100 bp ladder, + control: positive control, - control: negative control.

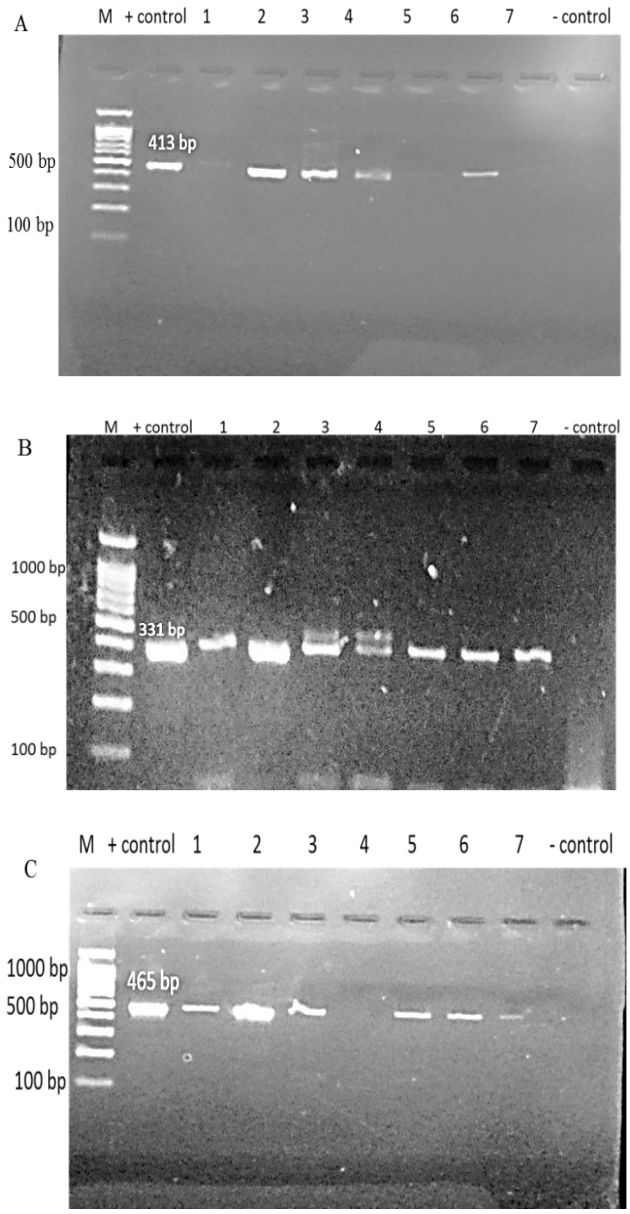

Fig. 4. Detection of *Bipolaris maydis* in plant debris during second round of nested PCR with primer pairs JB588/JB591(A), JB587/JB596(B) and JB588/JB598 (C) amplifying 413 bp, 331 bp and 465 bp bands respectively. Lane 1-7: First round PCR product from plant debris with general primers ITS1 and ITS4, labeled as PD1, PD2, PD3, PD4, PD5, PD6 and PD7. M: 100 bp ladder, + control: positive control, - control: negative control.

Climatic conditions are not the single most factors influencing disease epidemic. Other probable factors attributed to variable disease severity ratings include inoculum levels and cropping pattern of the area (Hooker, 1970; Harlapur *et al*., 2000; Kar, 2006; Harlapur & Utpal, 2013). Varieties planted in an area, farmer education and management, strategies adopted by the growers also affect disease distribution pattern. Probably, the growers of the highland wary of disease occurrence because of their previous experience and had opted for resistant varieties or the use of additional fungicides to protect their crop. Although farmers were not interviewed yet the low distribution of the disease in the highland of the province substantiated this hypothesis.

The present study has identified hot spots of the disease in diverse agro-ecological areas of KP which emphasizes the need of in-time disease management strategies to avoid the risk of epidemics in such areas. An integrated disease management strategy with full emphasis on use of genetically diverse varieties, removal of plant debris, crop rotation, good agronomic condition to improve micro-climate and rational use of fungicides to avoid emergence of fungicide resistant races should be adopted in the area.

In the present study, direct PCR for detection of the pathogen in asymptomatic leaves and nested PCR in infected plant debris were used. Such a protocol is an alternative to conventional methods for detection.

Moreover, this protocol could detect the pathogen in maize tissue with mixed infection since the primer pair is species specific and can readily detect the pathogen with reasonable sensitivity.

Initially three primer pairs including JB588/JB591, JB587/JB596 and JB588/JB598 were used to detect the pathogen both in asymptomatic leaves and plant debris through direct PCR. Amplification bands of various sizes were observed when DNA was amplified from asymptomatic plant leaves. However, target DNA of the pathogen was not amplified in the samples containing plant debris with direct PCR. This may be due to the presence of PCR inhibitory compounds such as phenol in plant debris, which are produced frequently as the decay progresses. Atkins & Clark (2004) categorized these inhibitors as an important impediment in reliable detection of pathogens through PCR from plant tissues. Therefore in an effort to counter this effect a nested PCR was used to detect the pathogen in crop debris. Compared to direct PCR, nested PCR is a sensitive assay eliminating the risk of contamination by diluting the first round PCR products before the next round (Bhat & Browne, 2010). Amongst all primer pairs, JB588/JB591 and JB587/JB596 out-performed primer pair JB588/JB598. Primer pair JB588/JB598 however failed to detects the pathogen in some samples which were previously identified by the other primer pairs. Similarly, primer pair JB587/JB596 was found excellent in detecting the pathogen in plant debris followed by JB588/JB598 and JB588/ JB591.

Conclusions

The study concluded that southern corn leaf blight was widely distributed throughout Khyber Pakhtunkhwa province. Incidence and severity of the disease was higher in plains including Peshawar, Nowshera, Swabi, Charsadda and Mardan than the highlands. Conventional PCR was successfully used for detection of the pathogen in asymptomatic leaves. Nested PCR was however necessary for reliable detection of the pathogen in plant debris. Moreover, PCR assay confirmed presence of the pathogen in plant debris. It is therefore suggested that these shall be ploughed under or burnt to minimize chances of disease onset.

Acknowledgements

The authors acknowledge Higher Education Commission for providing financial support during the completion of the study.

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(Received for publication 24 October 2019)