MOLECULAR IDENTIFICATION OF XANTHOMONASAXONOPODIS PV.MALVACEARUM(XAM) ASSOCIATED WITH BACTERAL BLIGHT OF COTTON.

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Abstract: Cotton (*Gossypiumhirsutum*L.) is crucial fiber and cash crop in world including Pakistan and responsible for the fifth most cultivated area in the world. It is the backbone of national economy and major source of foreign currency earning in Pakistan. Bacterial blight of cotton, caused by *Xanthomonasaxonopodis*pv. *malvacearum*is a severe disease of cotton worldwide, which is the main source of qualitative and quantitative losses in several cotton growing vicinity of the world and also in Pakistan. Twenty advance varieties/lines names are F.H-312, F.H-941, F.H-118, F.H-114, MNH-886, F.H-942 (non BT), LalaZar, F.H-142, MS-71, MNH-882, MS- 289, CM-595, FH-682, FH-900, Z-31, CA-12, Ali Akbar-703, Huma-15, Ali Akbar-703-905 and N-112 of cotton were grown under field conditions in research area of Department. The resistance capability was recorded according to the disease rating scale. These varieties/lines were tested against *Xam*, for its isolation and purification. Out of twenty varieties twelve were moderately resistant, three were moderately susceptible, three were susceptible, and two were highly susceptible. Gram staining, 3% KOH and artificially inoculation tests were used as pathogenicity. For the isolation of DNA and its molecular detection was done through PCR by using BOXIR Primer and also checked its diversity analysis rep-PCR. *Xam* showed the DNA size 300bp-1.5kb compared with 1.5kb ladder or marker. The bacterial titer which was assessed through PCR and its value was equated with the visually observed symptoms to evaluate the plant response.

Introduction

Agriculture in Pakistan is the lifeblood of the nation. It is the largest zone of the economic activity and plays a central role in the country's economic growth by providing food and raw materials and employment to a large proportion of the population. While agriculture grips an important place in Pakistan's economy, its contribution of 21 percent to GDP, 43.7 percent to the total labor force and a major source of foreign exchange earnings (GOP, 2014). Cotton (*Gossypiumhirsutum*L.) is crucial fiber and cash crop in world including Pakistan.). In the world, Cotton is cultivated in more than 80 countries (Sunilkumar*et al*., 2006). It is responsible for the fifth most cultivated area in the world. It is the backbone of national economy and major source of foreign currency earning in Pakistan. It is grown over fifteen percent of the country's arable land. It accounts 8.6 percent value added in agriculture sector, 55 percent to the foreign exchange earnings and about 1.9 percent to GDP of the country. In 2014-2015 Pakistan produce 13,983 thousand bales (GOP, 2015). In Pakistan, It adds about 55% of the total edible oil production (Shah *et al*., 1999). Cotton is the world peak widely sown fiber crop, has an important share in global economy (Cuming *et al*., 2015) and a significant contributor of oilseed with an approximate utilization of about 115 million bales (Waqas*et al*., 2014). In Mexico scientists found pieces of fabric made by cotton cloth that were at least 7,000 years old (http:// www.sewalot.com/history_of_cotton.htm). Several biotic factors influence the yield of crop like weeds, infestation of insects, pest attack like bacteria, fungi and viruses etc. and abiotic factors like deficiency of water, lack of nutrients, relative humidity and high temperature but diseases also playing an important role in yield reduction due to effect on germination of plant, decreasing plant productivity and lint quality. In Pakistan following diseases; Cotton leaf curl virus

(CLCuV) by Gemini virus, Wilt caused by *Verticilliumdahliae*, Root Rot which is caused by *Rhizoctoniasolani*and bacterial blight caused by *Xanthomonasaxonopodis*pv.*malvacearum*are most important.

The genus *Xanthomonas*is the most important and falls in the top ten plant pathogenic bacteria among them. Almost a 100-year history of research has contributed to the current understanding of the pathogenicity of the different *Xam*races. Bacterial blight of cotton was first recorded in the USA. (Atkinson 1891). Knight (1948b) proposed the disease may have originated from India. This pathogen caused 73,000 bales loses due to foliar and stem phases of this disease in USA projected by Cotton Disease Council (Watkins, 1981). During 1950s the disease became dominant in the USA (Schnathorst*et al.*, 1960) and in the 1970s in India (Verma, 1986).

In Pakistan it was reported near Multan in 1965 (Evans, 1965). In most cotton growing area of Multan 50% infection was recorded (Ali, 1968). The bacterium does overwinter on plant residue infected lint, and seed left in the field after harvest. The bacterium can survive at least 4 months on the seed lint and 22 months on the seed (Kirkpatrick and Rothrock 2001). Sprinkler irrigation, flowing water, wind-driven rain, and rain splatter are the primary ways to spread *Xam*(Brown and Ware 1958; Brinkerhoff 1970). Khan and Illyas (1990) inoculated the plants 20 days old artificially over noninoculated control and resulted 41.7 % seed cotton yield reduction.

Atkinson (1892) stated bacterial blight of cotton and defined its particular causal body. Originally, the responsible bacterium for CBB was named as *Pseudomonasmalvacearum*, then bacterium *malvacearum* (Dowson 1939),*X. campestris*pv. *malvacearum* by Dye *et al*.,(1980)and *X.axonopodis*pv. *malvacearum*(Vauterin*et al*. 2000). The pathogen is presently named *X. citri*pv. *malvacearum*, based on DNA analysi of the 16S-23S ribosomal intergeneric spacer sequence (Schaad*et al.*, 2006, 2007).

Stoughton (1930, 1931,1932 and 1933) performed an experiment to study the effect of humidity and temperature bacterial blight of cotton for this he used simple growth chambers and concluded that infection take place most readily when high air temperature (between 32^{o}C and 36^{o}C) is combined with high atmospheric humidity. Weindling, (1948) revealed that varieties of Egyptian and sea-island*(G.harhadense*L.) were more susceptible to the disease than varieties of uplandcotton(*G.hirsutum*L.). Schnathorst*et al*., (1960) conducted a research on history distribution, races, and disease cycle of *Xam*in California and stated that overhead sprinkler would promote splashing over the entire plan. According Schnathorst and Halisky, (1960) it enters in plant through wounds or natural openings. Brinkerhoff, (1970) studied in Punjab severe attack of rust reduces the yield and the losses are more due to humidity and rains favor disease development, ranges from 1 to 27%, depending on the cultivar and crop age (Mishra & Krishna 2001). There were four races of pathogens viz. race 8, 10, 12, and 18 were recognized in most of the cotton growing areas of Punjab. Race 18 was more dangerous strain of bacteria in the world and has been also recorded from Pakistan (Hussain, 1984). (Kazemi-Pour *et al*., 2004) stated that extracellular enzymes directly or indirectly affect the bacterial population and symptoms in the host.

Since then, computer-assisted analysis and DNA homology have become the main techniques used to classify bacterial species. In 1997, *Xam*was reclassified into species *Xanthomonasaxonopodis*based on DNA hybridization experiments (Vauterin and Swings 1997). A combination of the results of DNA hybridization analysis and REP-PCR also supported this

classification(Rademaker*et al*. 2005). Abdo-Hasan *et al*., (2008) conducted an experiment and studied that molecular characterization of Syrian races of *X. citri. malvacearum*DNA was isolated and purified from bacterial cells, concluded that using RAPD and ISSR is a suitable typing method for analysis of genomic DNA, for determining genetic relationships among *Xcm*isolates both these techniques proved to be fast, reliable and sensitive. For molecular characterization isolated the DNA for identification of *Xam*through rep-PCR additionally used SDS-PAGE to determine extracellular activity and checked its genetic diversity. Rep-PCR fingerprinting indicated that the HVS was very closely related to other strains of *Xam*by Huang *et al.,* (2008). Alexander, (2009) performed an experiment on isolation frequency of *Xam*, by acid delinting and easiflo treating cotton seed and molecular detection of Xam through PCR, concluded that development of bacterial blight vary greatly by the type of inoculation used. Zhai*et al*., (2010) stated REP-PCR revealed that HVSs ensured a close genetic relationship with race 18. Chakrabarty*et al*. (1997) identified two avr/pth genes, pthN and pthN2, in HVS XcmN, a strain isolated from Burkina Faso that is virulent on all differential cotton lines including 101-102B. Although many *Xanthononad*genomes have been sequenced (da Silva *et al*. 2002; Qian *et al*. 2005), only 59 nucleotide sequences of *Xam*have been deposited into the NCBI database.

Razaghi*et al*., (2012) conducted an experiment on characterization of *Xanthomonas*citri subsp. *malvacearum*strains in Iran. In plasmid analysis, using alkaline lysis method, a single plasmid was detected for all examined isolates with molecular weight of 23 kb. Repetitive sequence based PCR (rep-PCR) fingerprinting assay using primer BOX AIR also failed to distinguish the Iranian isolates.Cunnac*et al.,* (2013) conducted an experiment on the sequencing of *Xam*and reported high-quality draft genome sequence of two strains (race 18 and 20) of *Xa*m.

Mondal and Shanmugam (2013) performed an experiment on advance methods for the detection of bacterial plant pathogens and stated that early finding in seeds and ensuring disease free planting materials through rapid diagnostics was likely the effective means of reducing bacterial disease incidence. They used PCR, nucleic acid based techniques and serological based diagnosis for bacterial diseases of crop related to seed.

Midha and Patil (2014) conducted a research on genomic evolutionary origin of *Xanthomonasaxonopodis*pv. *citri*(*Xac*) clear thoughtful of the phylogenetic andevolutionary relationship of a complex group of bacteria like *Xanthomonas*needed to build a sense of the variations occurring during the diversification of its members into different lineages and sub-lineages. Showmaker*et al*., (2014) performed an experiment on genome sequence of *Xam* and stated that genome sequencing of *Xam*facilitated the researchers to develop a specific PCR assay for the revealing of pathogens from plant materials based.Ajene*et al.,* (2015) conducted an experiment and studied the biochemical and molecular detection of *Xam*pathogen, casual organism of bacterial blight of cotton in northern area of Nigeria. CR was performed for molecular detection of these isolates which positively identified the pathogen. The bacteria reacted positively to Tween 80 and digestion of arabinose, cellobiose, arabitol, acetate and lactate were positive.

Asgarani*et al*., (2015) performed a comprehensive study on sequencing and fingerprinting of *Xanthomonas*spp and stated that species were recognized by sequencing of 16S rDNA region using universal primers enclosing 27F and 1492R. Sequencing results were assessed by bioEdit version 7.0. 9.0 program and also done ERIC-PCR, yielded 5 to more than 21PCR products, ranging in size from 100 bp to over 5 kb.

Jalloul, *et al*., (2015) conducted an experiment on bacterial blight of cotton and found that the interactions between host plants and bacteria are based on the gene-forgene concept, which indicating a complex resistance gene/avr gene system and molecular identified it through PCR at gene level.

This matter in Pakistan has embraced the severity due to unawareness of all manufacturers and the contribution of none technical persons in pesticide marketing and poor and non-judicious use of pesticides has mounted the problems of that issue beyond the standard methods and recommendations. Systemic infection is an exciting issue to control the bacterial blight. Moreover, habit of synthetic chemicals for managing the diseases was discouraged recently due to their toxic nature and cause environmental pollution. According to Salah Eddin*et al*., (2007) this problem is controled by cultural practices like destruction of crop residues, crop sanitation and crop rotation, chemical seed treatments and foliar sprays and breeding of resistant varieties. But presently none of the high yielding commercial varieties has a durable resistance against the disease (Hussain *et al*., 1985; Rashid and Khan 1999). Little or no evidence about blight resistance is available for many of the cultivars manufacturing by private companies (Bayles and Verhalen, 2007). Any control methods that can reduce the yield losses due to disease will significantly contribute to the country economy. Timely detection of this bacterial pathogen is extremely important in forming management strategies against the disease of bacterial blight of cotton.

To improve our understanding of the cotton-*Xam*association, there is a serious requirement to understand the genetic and biochemical role that R-genes play to put heads together resistance to *Xam*. To examine this goal and the underlining molecular mechanism(s) causing bacterial blight symptoms and host plant resistance, bacterial blight resistance genes are needed for high resolution mapping and cloning (Yang, 2013).

The major purpose of this research is to find out the bacterial blight pathogen on 20 of cotton varieties/lines which are normally grown for lint purpose in the country. A comprehensive study on the bacterial blight of cotton, isolation and identification through PCR was done.

Methodology

Sowing of genotypes: For the establishment of diseased screening experiment of cotton crop against *Xam*the cotton seed were collected from Department of Plant Breeding and Genetics University of Agriculture Faisalabad, Cotton Research Department of AARI Faisalabad and from NIAB Faisalabad and tagged these seed according to that variety/line name. These varieties/lines were sown in the Department of Plant Pathology research field in during the cotton growing season of 2015 using RCBD design. The names of twenty varieties/lines are as F.H-941 and F.H-942 N, F.H-LalaZar, F.H-114, F.H-118, N-112, F.H-312, F.H-142, Aliakbar-703, MNH-886, F.H-682, MNH-882, F.H- 900, MS-289, CM-595 and Z-31, Ca-12, Aliakbar-950 and Huma-15. Infected leaves were collected from disease affected field. Leaves were collected on visual symptoms related to *Xanthomonasaxonopodis*pv.*malvacearum.* Nutrient agar media (NA) was used for isolation, multiplication and purification of bacterium. Beef extract 3.0g Glucose peptone 2.5g, Agar 5.0g and Distilled water 1liter Above mention all ingredients were weighed one by one (exept water) on digital balance and were poured in a sterile flask. Distilled water was added and after that volume was made upto 1000 ml and autoclaved the media.

Isolation from infected leaves: Leaves exhibiting bacterial blight symptoms were collected from field trails and were taken to the laboratory. Bacterium was isolated by dilution plate technique (Clifton, 1958). Leaves having spots were cut into small pieces. These pieces were dipped in 70% ethanol up to one to two minutes in order to disinfect the saprophytes present on the surface and put them on Petri plate for growth and further evaluation. Incubate these samples Petri plates for 24 hours. Following pictures clearly shown the *Xam*growth on NA media, Yellow colors Round colonies are visible.

Pathogen identification through biochemical and diagnostic tests:These tests were performed to confirm the *Xanthomonascampestris*pv.*malvacearum*. As *Xam*is gram negative bacteria for this conformation following tests were performed.

1- **3% KOH:** The gram staining results were confirmed with a 3 % KOH test. For this, 3% KOH solution drop were placed on slide and bacterial mass was mixed continuously for 1 minute with pipette and pulled the loop away from the slide loop needle. Loop was formed which means that bacteria was gram negative bacteria (Ryu, 1940).

2- **Gram Staining:**Gram staining was done by preparing a bacterial smear on slide with a loop, dried and heat fixed through the spirit lamp flame. The dime of slide was treated for 40 seconds with 0.5 % crystal violet, washed then treated with iodine solution for 30 seconds and decolorized with 95% ethanol. The slide again stained with 10 % safranin solution for 50 seconds and viewed at 100 X magnification (Gehardt, 1981).

Inoculation in healthy plants: For preparation of inoculum, the nutrient broth media was used. For initial inoculum preparation 5 ml of the nutrient broth was taken in a sterile test tube and were inoculated with the 50ul of bacterial isolate. When pure colonies were fully grown on Petri plate, an aqueous suspension of bacterium 108cfu/ml was prepared by dilution plate technique method. Thus pure colony of isolated bacterium was prepared for inoculation and pathogenicity test was performed. Plants with good health were sterilized with distilled water. The method for leaf inoculation was used by rubbing, syringe followed by spray for pathogenicity test to confirm Koch's postulates shown in figures. Non inoculated plants served as control. Daily observation and recording of the data was carried out to check the symptoms or response of plants. Pathogenicity tests were conducted in controlled conditions to verify the identification and pathogenicity of the isolated bacterium. The trial was conducted on the susceptible variety CM-595. The bacterium was re-isolated from diseased leaves after 8 to 10 days of inoculation by dilution plate technique. Morphological characteristics of re-isolated bacterium were compared with bacterium culture that was inoculated. The re-isolated bacteria showed exactly same colony characteristics as that of original culture were considered to be pathogenic.

Screening of Genotypes: Varieties were screened as immune, resistant, moderately resistant, susceptible and highly susceptible according to the Brinkerhoff (1977) disease rating scale. The data of disease incidence was collected on weakly basis on seedling flowering and at boll formation according to disease rating scale. Disease incidence was calculated by using formula (Jagtap*et al*., 2012).

$$\text{Disease Incidence} = \frac{\text{No. of infected Plants}}{\text{Total No. of Plants}} \times 100$$

Statistical Analysis: The data obtained with respect to diseases incidence was statistically analyzed by using SAS/STATE software (SAS Insitute, 1990) with least significant design (LSD) at 5% probability level (Steel *et al.,* 1997).

Genomic DNA Isolation: Genomic DNA was isolated by the method described by Kronstad*et al.*, (1993) with slight modification. 1mL of bacterial culture was inoculated in 100mL LB broth, incubated in shaking incubator at 600C and 100rpm for 24 hours. The overnight grown fresh culture was centrifuged at maximum speed (14000 rpm for 5 min), the supernatant was discarded and pellets were resuspended by vigorous vortexing in 500 µl TE buffer to which 60 µl 10% SDS and 60 µl proteinase K (20 mg/ml) were added. After 1h incubation at 37ºC, DNA was sheared by 3-5 passages through a 36G needle and extracted twice with phenol/chloroform (1:1) and twice with chloroform. Nucleic acids were precipitated using absolute ethanol and sodium chloride. The resulting pellet was resuspended in TE buffer containing RNase A and incubated at 37ºC for 30 min. DNA was finally precipitated with 5M ammonium acetate and isopropanol. After centrifugation, the pellet was washed twice with 70% ethanol,air dried and dissolved in TE buffer. (40 mMTris-acetate, pH 8.0 containing 2 mM Na2-EDTA) at 100 volts for 30 min. The gel was stained with silver nitrate (0.5µg/ml) and the plasmid DNA bands were visualized with a UV transilluminator. DNA samples and the DNA standard marker were loaded into the wells of the solidified gel submerged in 1X TAE. Gel Electrophoresis was carried out 80 volts until the required DNA separation was estimated to be achieved. The DNA bands in the gel were visualized using short wave ultraviolet light provided by a transilluminator and photographed using Dolphin-DOC gel documentation system (WEALTEC). For PCR and rep-PCR used following protocol Taq polymerase 0.3ul, Taq Buffer 2.5ul, Dntps 1.0ul, Mgcl2 1.5ul, Primers 2 x 0.5ul (reverse & forward)Template DNA 2.5ul, Ddd H_2O 16.2ul.

DNA extraction and running rep-PCR

The DNAs from *Xcm*strains were amplified with primer of BOX AIR (5 'CTACGGCAAGGCGACGCTGAC 3')according to Seal *et al*. (1993) and Razaghi*et al*., (2012).REP (REP1R: 5′-IIIICGICGICATCIGGC-3′, REP2I: 5′-ICGICTTAT CIGGCCTAC-3′) each with their specific annealing temperature Versalovic*et al*., (1994). Box electrophoresis of polymerase chain reaction (PCR) fragments were separated by electrophoresis on agarose gel (0.5%), stained with ethidium bromide (0.3 µg/ml) and photographed under ultraviolet (UV) light. PCR standard marker deoxyribonucleic acid (DNA) phage lambda was used with bands at defined intervals of 1500, 1200, 1031, 900, 800, 700, 600, 500, 400, 300, 200 and 100 bp.

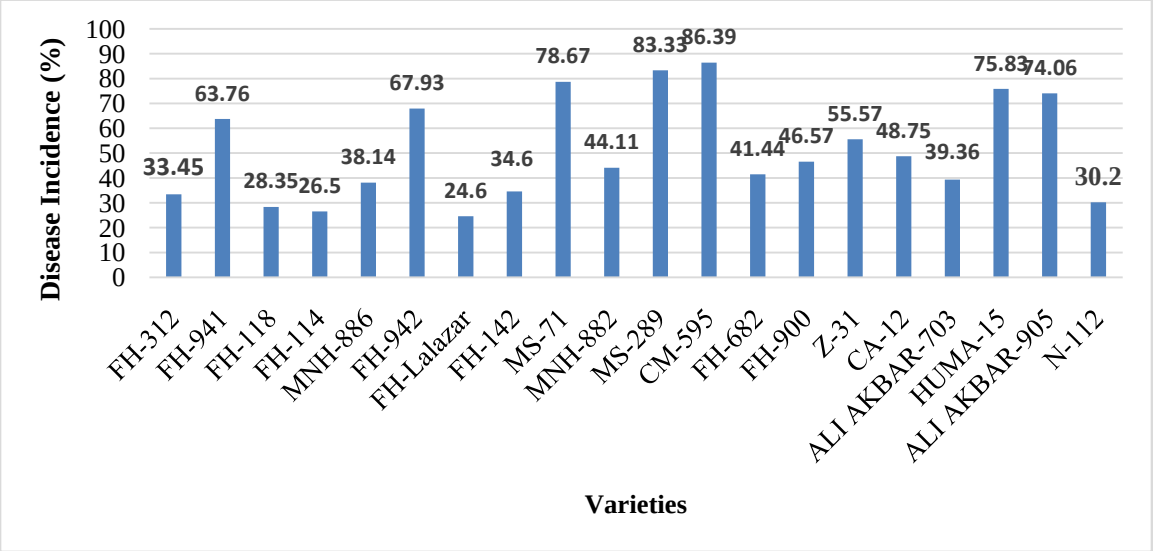
Results:

Screening of varieties/lines against *Xanthomonasaxonopodis* pv. *malvacearum.*

Sr.#	Varieties/ Lines	Disease rating	Disease incidence (%)	Response
1	F.H-LalaZar	4	24.600t	MR
2	F.H-114	4	26.500s	MR
3	F.H-118	4	28.350r	MR
4	N-112	4	30.200q	MR
5	F.H-312	4	33.450p	MR
6	F.H-142	4	34.600o	MR
7	Aliakbar-703	4	36.360n	MR
8	MNH-886	4	38.140m	MR
9	F.H-682	4	41.437l	MR
10	MNH-882	4	44.110k	MR
11	F.H-900	4	46.570j	MR
12	CA-12	4	48.750i	MR
13	Aliakbar-905	5	55.560h	MS
14	Huma-15	5	63.767g	MS
15	MS-71	5	67.930f	MS
16	MS-289	6	74.060e	S
17	CM-595	6	75.830d	S
18	Z-31	6	78.670c	S
19	F.H-941	5	83.333b	HS
20	F.H-942 N	5	86.390a	HS
LSD = 0.3618				

All 20 means are significantly different from one another.

4.2. Graphically Response of Varieties



Graphically response of Varieties with means value

Symptoms revealed the clear image of disease incidence by which we can easily recognise the disease. The symptoms apeared in the form of water soaked spots which were formed as a result of pathogen attack. These symptoms became dark brown with the passage of time and spread on the ventral surface of young leaves. A significant difference was observed among cotton germplasm in terms of disease incidence. Disease incidence ranged from 24.6 to 86.39 percent. Themoderately resistance, moderately susceptible, susceptible and highly susceptible varieties are shown in table.

Among twenty varieties F.H-LalaZar, F.H-114, F.H-118, F.H-118, N-112, F.H-312, F.H-142, Aliakbar-703, MNH-886, F.H-682, F.H-900 and CA-12 showed moderate resistance to bacterial blight with disease incidence ranging from 24.6 to 48.7%. F.H-LalaZar showed minimum response against *Xam* with disease incidence 24.6 percent which is shown in table and also in graph. While other three varieties Aliakbar-905, Huma-15 and MS-71 showed moderately susceptible response with (55-67%) disease incidence. In these moderately susceptible varieties, Aliakbar-905 was showed moderately response against *Xam*with disease incidence 55.760. The varieties MS-289, CM-595 and Z-31 with (74-78%)disease incidence showed moderately susceptible. The varieties F.H-941and F.H-942 N showed highly susceptible with (83.33-86.39%) disease incidence against the disease and rated as 5 in the above table. All of the studied cotton varieties showed a varied degree of resistance against bacterial blight of cotton. It was noted that no variety was completely resistant to bacterial blight of cotton. However twelve varieties were found moderately resistant.

Molecular Characterization of *Xam:* For the molecular identification of *Xam*, genomic DNA was extracted using (CTAB) method. Thermal cycler was used for PCR amplification and specific primers BOXIR were used which amplifies the specific 300bp fragment.(Seal *etal*. 1993;

Razaghi *etal*., 2012). Six samples were used for DNA isolation. There were some impurities in genomic DNA which shown in 3 sample, for removal of those impurities phenol chloroform was used. Gel electrophoresis was used for DNA confirmation.

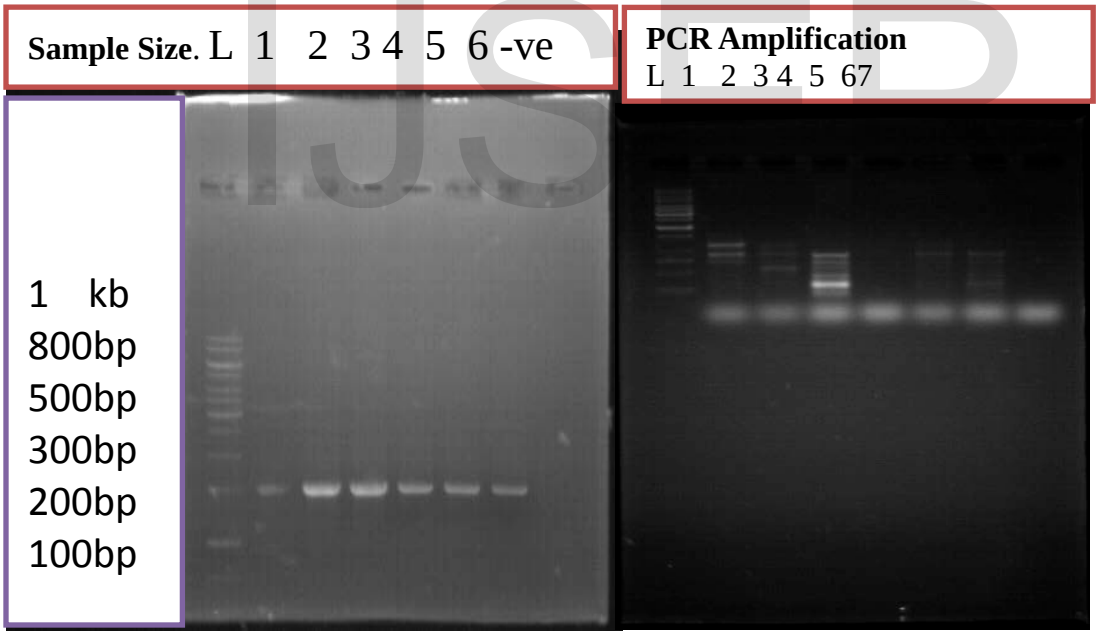
Polymerase chain reaction (PCR):

DNA component of *Xam* was amplified by using BOXIR [forward primer---CTACGGCAAGGCGACGCTGAC---] and [Reverse primer---GATGCCGTTCCGCTGCGACTG----.] primers were design to amplify a specific region. PCR product of 300bp size was successfully amplified in all samples, which are equal to sizein **Fig.**For confirmation amplification of *Xam*, in six samples were loaded with one negative. The negative have no DNA due to which it was not amplified. 1kb ladder was used for the amplification and fig. clearly showed that our DNA was amplified at 300bp region**.**

Rep PCR

Genetic diversityby rep-PCR with BOX primers was performed on four isolates. The genomic rep-PCR profiles contained bands ranging in size from 300bp to 1.5 kbp (Fig. The patterns obtained by rep-PCR analysis were identical for all tested strains. This figure showed the genetic diversity of *Xam*, intergenic spacer sequence of DNA genome.

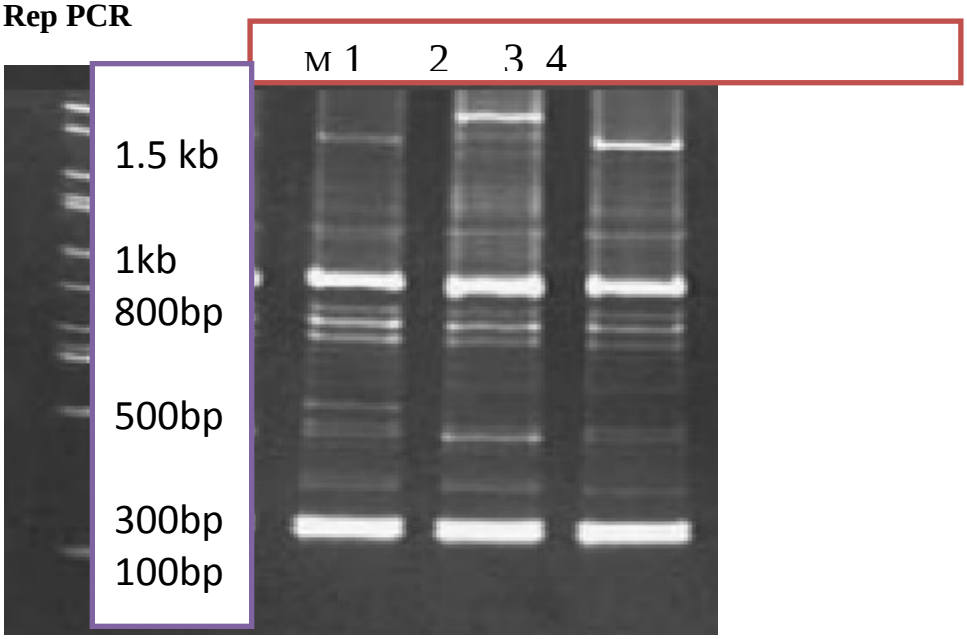
Gel Documentation



Genomic DNA was isolated from seven samples

Rep- PCR

Rep PCR



Discussion:

Cotton is most widely cultivated crop in world and very famous for research purpose because cultivars from upland cotton species fulfill 90% of bulk world's cotton demand (Wendel*et al.*, 1992). Cotton is significant because it is fiber crop used in textile industry and it is also important due to import and export (Islam *et al.*, 2003). Its area under cultivation is about 32.3 million hectares worldwide with annual production 113.9 million bales (USDA, 2010). Pakistan's annual cotton production is 2,129 thousand tons from area of 2,689 thousand hectares. Cotton is grown for its textile importance and also its seeds are fulfilling 4% world's vegetable oil demand (Bruinsma, 2003). Although specific to a certain *Xam* race, resistance deliberated by a single dominant Rgene overcome by the pathogen. So far, the formation of pyramided Rgene-containing lines effective against all races of *Xam*has not been achieved(Jalloul*etal.*, 2015).

Bacterial blight of cotton (BBC) caused by *Xam*is a major hazard to the cotton among the diseases (Vauterin*et al*., 2000).The pathogen *Xam* enters the host plant through either the stomata or wounds and creates water-soaked lesions on leaves, stems and bolls, followed by premature leaf senescence and reduced lint yield (Rungis*et al.*, 2002).About 10 to 30 percent losses due to this disease were reported by (Kalpana*et al*., 2004). Yield losses due to this pathogen depends upon the cultivar and age of the crop plant (Mishra and Krishna, 2001). Yield losses were high as 60% resulted under Central Asiatic conditions and 36% caused by two stages blackarm and angular leaf spot of bacterial blight of cotton (Serbinoff, 1934).

Resistant varieties were introduced successfully in late 1990s, especially in the Punjab, and their cultivation was successful for several years. Plant disease resistance is characterized by complete or partial suppression of the pathogen growth and its replication at infection site (Bhuiyan, 2009). There is mechanism behind in each type of resistance which supports the plants population to perform action against virulent pathogen. When *Xanthomonasaxonopodis*pv.

*malvacearum*enterd into mesophyll cells of cotton leaves they showed necrotic lesions due to the host cells death response against bacteria which prevented them from further infection. This type of response is called hyper sensitive response (HR). In resistant varieties of cotton the HR mechanism is not compulsory to initiate with combination of pathogen and host it required a source of energy in the form of ATP, this source of energy responsible for efflux of $K^+$and influx ofH^+ increase in that area where bacteria attacked (Gotto,1992).

To find out the sources of resistance 20 genotypes of upland cotton were assessed against *Xanthomonasaxonopodis*pv. *malvacearum* under natural environmental conditions, any of them were not free of *Xanthomonas*. None of the variety was immune, 12 were moderately resistant, three were susceptible, three were moderately susceptible and two were highly susceptible in their response against the pathogen. Ramachandran and Ramakrishnan (1950) documented that *G. arboretum* and *G. herbaceum* cultivars were found protected and unaffected to this disease, while *G. barbadense*and *G. hirsutum* are vulnerable. This shows overall variation in the genetic makeup of the varieties of upland cotton and improvement is required in this direction.

The variety Reba-B50 was possessed resistant against BLB when experiment was conducted by Singh and Verma (1971). In this research F.H-LalaZar, F.H-114, F.H-118, F.H-118 projection moderately resistant varieties of cotton were determined. The sensitive lines were Aliakbar-905, Huma-15 and MS-71 etc. The other five varieties are disposed of in the field under natural field conditions.

Hussain *et al*., (1984) had completed a screening test at the Central Cotton Research Institute (CCRI), Multan and observed that ten lines of exotic cotton presented by the United States were immune to all the races here in *Xam* in Pakistan, segregating one, seven other highly susceptible and sensitive. None of the lines of local strain were immune against the pathogen. In this study none of the variety shown immunity against *Xam*.

Atiq*et al*., (2014) conducted research on fifteen varieties screening against cotton bacterial blight and resulted that F.H-114 and CIM-595 shown moderately resistance against this pathogen. In present research F.H-114 is shown moderately resistance response against it but CIM-595 shown susceptible response against this pathogen.

For a long time a large number of *Xanthomonas*strains have been characterized by a variety of phenotypic and genotypic methods in a multitude of studies. Among these, genetic studies employing DNA-based techniques have played an important role in determining the genomic diversity and relationships within the genus, which has led to reclassification of *Xanthonomas* species. Aritua*et al.* (2007) raised some pathovars to species level, whereas other species were given a pathovar status. In this context, the causal agent of cotton bacterial blight long known as *Xanthomonascampestris*pv. *malvacearum*(Smith) Dye, was changed to *X. axonopodis*pv. *malvacearum*(Vauterin*et al.*, 1995, 2000).

Regarding the molecular characterization of bacterial blight pathogen of cotton in Pakistan, very few studies have been conducted so far (Chaudry and Rashid, 2011). They characterized negative bacteria for several tests and concluded that pathogen positive for gram staining and 3% KOH test. This research also revealed by 3% KOH that this pathogen is gram negative.

Duetoglobalhumanactivity,transportofplanthosts andvectorsbyglobal tradeandnew agriculturalpracticescoupledwithclimatechange,thediversity of bacteria and their races is increasing. Molecular based identification is best way to determine the genetic diversity of the *Xam* races. Huang *etal.,* (2008) revealed the identification of a highly virulent strain of *Xam* on the basis HVS gene (GSPB 2388) in Sudan by using BOXIR marker and showed the high virulence strain 18 DNA amplification through PCR and also for finger printing of *Xam.* Primers corresponding to conserved DNA sequences of REP elements, BOXA subunits of BOX elements, and ERIC sequences annealed to genomic DNA and generated unique genomic fingerprints. In this research, BOXIR primer is used for amplification through PCR. Result showed that *Xam* DNA amplification confirmed the BOX and PCR yielded same six PCR products, ranging in size from approximately 300 bp.

Genetic fingerprinting by rep-PCR with BOX primers was performed on nine isolates. The genomic rep-PCR profiles consisted of bands ranging in size from 100 bp to 1 kbp. The patterns obtained by rep-PCR analysis were identical for all tested strains. Razgi*etal*.,(2012). The present study was also represent that *Xam*genomic DNA diversity profile consisted of bands ranging in size 300bp, the patterns obtained through PCR in four isolates. These results suggest that the virulence of *Xam*highly influenced by genotype and or geoclimatic foundation of the strains (Zhai*et al*., 2010).

According to Ajene*et al.,* (2015) molecular detection of *Xam*pathogen performed through PCRwhich positively identified the pathogen. In present study PCR is also performed to detect the *Xam* pathogen in six samples to confirm the DNA by gel documentation of 300bp.

Huang *et al.,* (1980) studied the molecular detection of *Xam* and used REP, ERIC and BOX restriction markers and concluded that the genome sizes are between 100bp to 3kb which show the genetic diversity of this pathogen. Present study results are also carried out by BOX restriction marker and showed diversity in four samples amplified samples size are between 300bp to 1kb. Overall, no pronounced effect was observed from the addition of unknown isolates on the stability of the cluster analysis based on the BOX-PCR banding patterns of the reference strains. However, it is important to realize that the continuous addition of new isolates encompassing a large taxonomical and/or geographical diversity may lead to a minor shift of the reference framework.

Conclusion:

In conclusion, rep-PCR fingerprinting by means of the BOXA1R primer is a rapid, easy to implement and reproducible method that is appropriate for a high throughput of bacterium strains. It is a highly discriminatory technique that permits differentiation at the species, subspecies and potentially up to the strain level. In our opinion, this technique is a promising tool for the identification of bacteria originating from all kinds of environments. The study has elucidated important out,pending regarding bacterial blight status in Pakistan of main cotton growing areas. The study concludes that disease has developed as a common disease and causing huge losses. A primary assumption in this work was that greater DNA recovery reflected a more representative (diverse) sample of DNA from the microbial community. New tools to rapidly compare the DNA diversities of extracts are needed to better estimate the effectiveness of DNA extraction protocols. Strict measures should be adopted to avoid its access and further spread in country and attention must be assumed for its management.

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