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Development and Optimization of Molecular Technique for Diagnosis of Citrus Canker in Citrus Cultivars

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ABSTRACT

The conventional method for the detection of *Xanthomonas axonopodis* has been based on biochemical tests. A rapid and sensitive method for identification and detection of *Xanthomonas axonopodis* is required for management of citrus canker. PCR-based diagnostic test is appropriate for monitoring pathogen in a very short time compared to laborious, non-specific and expensive protocols. ELISA, Nested PCR has been used for many years in different countries. Citrus orchards in Sargodha region were surveyed and leaf samples showing typical symptoms of citrus canker were collected. Infected section of leaf was taken for isolation of bacteria. Lesions were cut into parts and streak bacteria by the help of inoculation loop grown in nutrient medium. Pure bacterial culture of *Xanthomonas axonopodis* were used for detection by standard PCR. *Xanthomonas axonopodis* was diagnosed by amplification of 16S rDNA. A fragment of ~1.4 kb was amplified and cloned for sequencing Out of three markers used (K1F-CIT/ K1R-CIT, K2F-CIT/ K2R-CIT, K3F-CUT/ K3R-CIY) K3F-CUT/ K3R-CIY gave best results repeatedly. So this primer pair can be used for identification/diagnosis of *Xanthomonas axonopodis.* Bacterial culture used as template in PCR and colony PCR gave better results as compared with extracted DNA from infected leaf.

Key Words: *Xanthomonas axonopodis citri,* Citrus canker, Molecular diagnostics

INTRODUCTION

Xanthomonas axonopodis is the most devastating bacterium causing citrus canker. Citrus bacterial canker occurs in citrus producing countries which are in the region of tropical and sub-tropical areas. Strains of *pthA, pthB,* and *pthC* susceptible to citrus canker show abnormal increase of cells in normal host range (Garbiel *et al.*, 2000). Canker effected plants show necrotic lesion that develops on leaves, fruits, and twigs. Surface of leaves becomes ruptured, shows blister appearance due to hyperplasia which converts it into spongy pustules. Citrus canker has astringent contagiousness on grapefruit, pineapple, limes and lemons and their hybrids. Monetary citrus cultivars are susceptible that must be removed when diseases are exposed (*Verniere, Hartung, Civerolo,* & *Pruvost*, 1998). Citrus canker is prevalent and has a challenging effect on reservoirs eradication.

Xanthomonas axonopodis citri, *Xanthomonas axonopodis compestris* and *Xanthomonas axonopodis aurantifolii* are gram-negative, rod like bacteria one flagellum. Size of flagellum is 1.5-2.0×0.5-0.75mm. *Xanthomonas axonopodis* have aerobic respiration and show obligate growth, yellow colonies are formed on culture medium. XAC have polysaccharide slime which helps them to form mucoid colonies; can grow in range of 25c-30c (Chand & Pal, 1982; Goto & Takahashi, 2000).

Economically, citrus fruit is considered very important. Chemical composition of essential oil extracted from citrus by the help of hydro distillation reveals six compounds i.e. limonene 80.51%, terpinene 6.80%, cymene 4.02%, ß myrcene1.59%, and pinene 1.20% (Jin & Suk, 2009).

Pakistan has attained 12th position in citrus production all over the world (Silva, 2013). In an area of 199 thousand hectares of citrus, Pakistan is producing 1832 thousand tons citrus is produced in Pakistan and the share of province Punjab is 95.10% and that of district Sargodha (Punjab) is 49.3% of total production in Pakistan (MINFAL, 2011-12).

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Due to attack of *Xanthomonas axonopodis* corky lesion on leaf and fruit, vein chlorosis, premature fruit falling, hyperplasia, sunken centre, and elevated margins are formed (Schubert, 2001). In January 2006, USDA has revealed that eradication of citrus canker is not possible. While in Florida one billion canker eradication projects were applied in ten years (Gottwald & Irey, 2007). Citrus canker is established as epidemic in Argentina, Oman, USA, Australia, Asia, Brazil, Saudi Arabia, China, Reunion Island (Schubert & Miller, 2000).

In recent years demand of citrus fruit is increasing, but *Xanthomonas axonopodis citri* is damaging crops at large scale. Sweet fruits are more susceptible for canker attack. . Eradication of crop helps to control incidence of citrus canker (Ribeirao & Machado, 2011).

Citrus canker is an alarming threat to citrus industry of Pakistan. *Xanthomonas axonopodis citri* (XAC) is the causative agent of citrus canker, prevalent in Subtropical citrus-growing areas. The disease can remarkably affect a wide range of citrus cultivars by defoliation, dieback, reduce fruit quality and pre-mature fruit drop and consequently is subjected to national eradication programs and international regulations.

MATERIALS AND METHODS

Marsh early (*Citrus paradisi marsh*), washington naval (*Citrus sinensis*), mid sweet (*Citrus maxima*), pineapple (*Ananas comosus*), kinnow (*Citrus reticulate*) tarocco (*Citrus aurantium*), pera rio (*Citrus paradisi Rio Red*), grapefruit (*Citrus paradisi),* kaghzi lemon (*Citrus limon*), and tarocco-N (*Citrus sinensis tarocco-N*) citrus cultivars were used in this study.

Bacteria isolation and colonies formation:

Bacteria were isolated from infected lesion of leaf and fruit after sterilization with 70% ethanol. Bacteria were oozed out after lesion crushing. Oozed bacterial colonies were cloned on nutrient agar plate (0.5% peptone, 0.3% yeast extract, and 0.5% NaCl, 1.5% agar in 1 liter (L) water) by sterilizing loop. Bacterial colonies were cultured in nutrient medium (0.5% peptone, 0.3% yeast extract, 0.5% NaCl in 1 liter (L) water).

Bacterial genomic DNA isolation:

Bacterial genomic DNA was isolated from culture medium by the help TE lysis buffer (100mM Tris pH 8.0; 2% SDS), Proteinase K, 5M NaCl (292 gm of Nacl +700ml H_2O), 24:1 chloroform: isoamyl alcohol, 0.5X TBE buffer(108gm Tris base+55gm Boric acid+40ml 0.5 EDTA: pH 8).

PCR based amplification:

PCR-based technique used for amplification of *Xanthomonas axonopodis* 16S rDNA, 23S rDNA region with the help of universal primers. Amplification was conducted in volumes of 25 µl in Peq STAR 96X universal gradient (PEQ Lab). Primers concentration and other condition were optimized to amplify DNA.

PCR reaction mixture:

PCR reaction mixture included $MgCl_2$: 2 µl, DNTPs: 0.5µl (invitrogen Corp, San Diego-C, USA), Taq buffer: 2.5 µl (Invitrogen Corp), Taq DNA polymerase: 0.2 µl (Invitrogen Corp), Culture medium: 3 µl, Forward primer: 1.5 µl (16S rDNA) K3F, Reverse primer: 1.5 µl (16S rDNA) K_3R, dd H_2O (13.8 µl)

- Initial denaturation: 94 °C for 1 minute followed by:
- Denaturation: 94 °C for 1 minute.
- Annealing: 50 °C for 2 minutes. X 26
- Extension: 72 °C for 3 minutes.
- Final extension: 72 °C10-20 minutes.
- Store: 4 °C (Above mentioned range of PCR keep constant while annealing temperature changed according to samples)

Confirmation of amplification:

Amplification of PCR product was confirmed by loading 3 µl of PCR product in 0.8% agarose gel prepared in 0.5X TBE buffer. Gel documenting system visualized amplification of 16S rDNA and about ~1.4 Kb fragment size was amplified.

Purification and ligation of PCR product:

PCR amplified product was purified through phenol chloroform extraction. Purified PCR product was ligated into PTZ57/RT cloning vector (Fermentas). Ligation mixture (insert, PTZ57/RT vector, $MgCl_2$, ddH_2o, ligase buffer) added into competent cell (prepared from protocol of Cohen et al 1992).

Screening of clones:

Isolated DNA was digested with appropriate enzymes using appropriate buffers to confirm clones either single or double digestion.

RESULTS

Bacterial DNA isolation:

Bacterial DNA was isolated from bacterial colonies on agar plate. Isolated genomic DNA was used for PCR amplification.

PCR amplification of 16S rDNA:

DNA was diluted for suitable PCR amplification. ~1.4 kb bacterial genome was amplified. Initially Three strategies, like using extracted DNA from infected leaf, colony PCR and PCR using bacterial culture was used. Better and

consistent results were obtained while using 3µl of bacterial culture as a template and by colony PCR method as compared to using isolated DNA from infected leaf. Three pairs of universal primers were used for amplification of 16S rDNA viz K1F-CIT/ K1R-CIT, K2F-CIT/ K2R-CIT, K3F-CUT/ K3R-CIY (Table 1). Out of three primers checked, K3F-CUT/ K3R-CIY gave better results (Fig., 1, 2).

Further, PCR conditions were optimized for diagnosis of *Xanthomonas axonopodis citri.* Annealing temperature of marsh early (*Citrus paradisi marsh*), washington naval (*Citrus sinensis*), mid sweet (*Citrus maxima*), pineapple (*Ananas comosus*), kinnow (*Citrus reticulate*) was 50.5°C for 2 minutes while for tarocco (*Citrus aurantium*), pera rio (*Citrus paradisi Rio Red*), grapefruit (*Citrus paradisi),* kaghzi lemon (*Citrus limon*), and tarocco-N (*Citrus sinensis tarocco-N*) had 50.2 °C annealing temperature for 2 minutes (Table I).

Cloning:

PCR amplified product was cloned in PTZ57/RT vector. Cloning product was screened by digestion with appropriate enzymes. ~1.4 kb DNA was confirmed on 0.8% agarose gel.

Table I: Optimization of primer for amplification

Forward primer	Reverse primer	Amplification
K1F-CIT GCATTTFATGACGCCATGAC	**KIR-CIT** TCCCTGATGCCTGGAGGATA	No
K2F-CIT CTTCAACTCAAACGCCGGAC	**K2R-CIT** CATCGCGCGCTGTTCGGGAG	No
K3F-CUT AGAGTTTGATCCTGGCTCAG	**K3R-CIY** ACGGCTACCTTGTTACGACTT	Yes

Table II: Optimized temperature for amplification of DNA.

Sr. No.	Samples Name	Temperature(°C)	Size
1	*Citrus paradisi marsh*	50.5 °C	~1.4Kb
2	*Citrus sinensis*	50.5 °C	~1.4Kb
3	*Citrus maxima*	50.5 °C	~1.4Kb
4	*Citrus aurantium tarroco*	50.2 °C	~1.4Kb
5	*Citrus paradisi rio red*	50.2 °C	~1.4Kb
6	*Citrus aurantifolia swingle*	50.2 °C	~1.4Kb
7	*Citrus sinensis tarroco-N*	50.2 °C	~1.4Kb
8	*Citrus paradise*	50.2 °C	~1.4Kb
9	*Ananas comosus*	50.5 °C	~1.4Kb
10	*Citrus reticulate*	50.5 °C	~1.4Kb

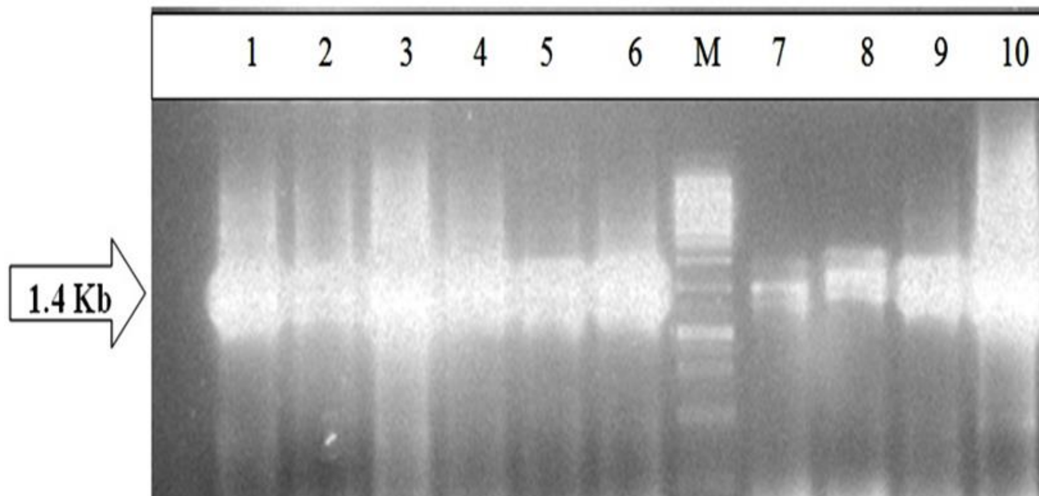

Fig., 1: PCR amplification from cultured bacteria

Lane 1 shows *Citrus paradisi marsh* amplified DNA, Lane 2 shows *Citrus sinensis* amplified DNA, Lane 3 shows *Citrus maxima* amplified DNA, Lane 4 shows *Citrus aurantium tarroco* amplified DNA, Lane 5 shows *Citrus paradisi rio red* amplified DNA, Lane 6 shows *Citrus aurantifolia swingle* amplified DNA, Lane M shows ladder (1000kb), Lane 7 shows *Citrus sinensis tarroco-N* amplified DNA, Lane 8 shows *Citrus paradisi* amplified DNA, Lane 9 shows *Ananas comosus* amplified DNA, Lane 10 shows *Citrus reticulate* amplified DNA.

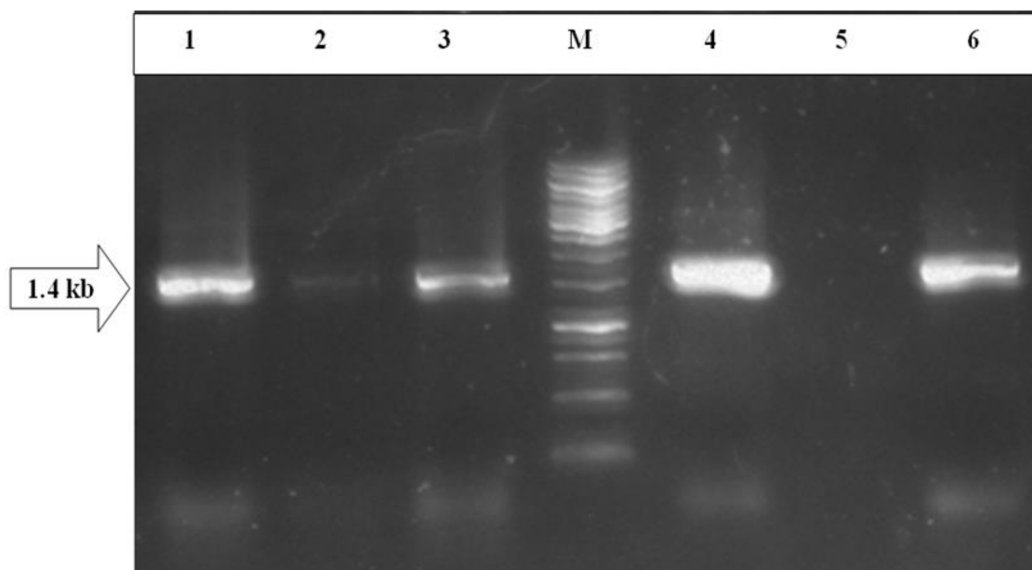

Fig., 2: Colony PCR amplification

Lane 1 shows *Citrus paradisi marsh* amplified DNA, Lane 2 shows *Citrus sinensis* amplified DNA, Lane 3 shows *Citrus aurantium* amplified DNA, Lane M shows ladder (1kb), Lane 4 *Citrus paradisi* amplified DNA, lane 5 negative controls, and Lane 6 shows *Citrus aurantifolia swingle* amplified DNA

DISCUSSION

Henson & French, (1993) used bio-assay method for recognition of *Xanthomonas axonopodis* strains. Different pathogenicity tests were performed but the results were not accurate while in current study it was observed that standard PCR provided accurate results for recognition of *Xanthomonas axonopodis citri*. Cubero & Graham, (2002) used extracted DNA from bacterial colonies for PCR amplification while in recent study purified colonies suspension was directly used for PCR amplification

and their clones were prepared. (Najafipour, 2014) used infected stem for identification of *Xanthomonas axonopodis* citri antecedently leaf was used for bacterial detection.

Ten citrus cultivars' were used for diagnosis of *Xanthomonas axonopodis* by PCR. All cultivars have different genetic makeup but they have receptors on conserved region. These receptors provide a site for attack of *Xanthomonas axonopodis.* Diagnosis of *Xanthomonas axonopodis* on varying cultivars reduces ambiguity about *Xanthomonas axonopodis* attack. It is the first ever study of using PCR method to diagnose citrus canker on molecular level from our local varieties in Pakistan and their clone's preparation.

CONCLUSION

Xanthomonas axonopodis attacks citrus cultivars on specific conserved region. Standard PCR provide accurate result for identification of *Xanthomonas axonopodis*.

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