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Detection and Testing Pathogenicity of *Xanthomonas axonopodis* pv. *punicae* Causing Bacterial Blight on Pomegranate

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ABSTRACT

Pomegranate, one of the most important fruit crops, is constantly challenged by Bacterial blight caused by *Xanthomonas axonopodis*pv. *punicae*, is a prevalent and destructive pomegranate disease. Accurate diagnosis of disease is very important to manage the disease. PCR have been widely used to detect or verify the presence of pathogen in recent decades. These molecular-based methods are rapid, accurate and sensitive for detecting pathogens. In this study, a primer set KKM5 and KKM6 was used and amplification of 491bp of *gyrB* gene proved the presence of *Xap*. The pathogenicity of the *Xap* was confirmed following Koch's postulates.

Introduction

Pomegranate bacterial blight (PBB) occurs in many pomegranate-producing tropical and subtropical countries around the world. It is a major problem in all areas of pomegranate grown in India. For the first time in India, bacterial blight of pomegranate was reported by Hingorani and Mehta (1952). Later on during 1959, Hingorani and Singh took a thorough investigation on the disease and causal organism and designated the pathogen

as *Xanthomonas punicae*sp. Nov. Chand and Kishun (1991) reported the epidemic form of disease in Bangalore caused 60 – 80 per cent loss during 1991. Later, on the basis of DNA homology, the pathogen has been renamed as *Xanthomonas axonopodis* pv. *Punicae* (Vauterin *et al.,* 1995). The development of rapid and reliable procedures for the diagnosis of this pathogen has been a priority.

The polymerase chain reaction (PCR) allows the rapid, specific, and sensitive detection of DNA sequences and thus is ideally suited to the detection of plant pathogens. We identified *Xap* using *Xap* specific primers developed by Mondal *et al.,* (2012) and pathogenisity of the pathogen was proved following Koch's postulates.

Materials and Methods

Isolation of the pathogen

The different parts of the pomegranate plant showing characteristic symptoms of bacterial infection were collected for isolation. Pathogen was isolated by streak plate method on NGA (nutrient glucose agar) media at 28°C. After 48 hours of incubation individual colonies are picked and sub cultured on media. Later pure culture is stored in refrigerator at 4° C for further use.

PCR and amplification conditions

To identify pathogen a primer set, KKM-5 Forward and KKM-6 Reverse designed by Mondal *et al.,* (2012).Total genomic DNA from *Xap* was isolated from the single colony inoculated in NG broth culture grown for 72 hr at 28±0.5°C.

Isolated DNA was quantified using the NanoDrop spectrophotometer (ND-1000, ThermoFisher, MA, USA). A primer set, KKM-5 Forward 5'GTTGATGC TGTTCACCAGCG3' and KKM-6 Reverse 5'CATTCATTTCGCCCAAGCCC3' were designed from the C terminus region of gyrB gene by Monda *et al.,* (2012b) was used for PCR-based identification of *Xap*. PCR amplification was performed in a 15 µl reaction mix containing 100 ng of DNA, 1X PCR Buffer, 200 µM of dNTPs, 0.2 µMoles each of forward and reverse primer and 1 U *Taq* DNA polymerase (Merck, Bangalore, India).

Primers were amplified with PCR cycle of initial denaturation at 94°C for 4 min, then 30 cycle denaturation at 94°C for 60 sec, annealing for 45 sec at 55°C and extension 72°C for 1 minute, followed by a final extension at 72°C for 5 minutes in a thermocycler (Eppendorfvepo protect Germany). PCR products were resolved on 1.4 per cent agarose gel using a horizontal electrophoresis system (Bio-Rad, Hercules, California, USA).

The amplified products were stained using ethidium bromide (0.001 mg/ml) and gel images were photographed using a Gel Logic 212 Pro imaging system (Gel Logic 212 PRO, Carestream, USA).

Testing the pathogenicity of *Xanthomonas axonopodis* pv. *Punicae*

Six month old Bhagwa plants raised in greenhouse conditions (28 ±0.5 °C, 60-70% relative humidity) were used for pathogenicity tests and for all other studies. Preparation of bacterial inoculum: Bacterial inoculum was prepared by inoculating the pure and single colony of *Xap* in NG broth, culture flasks were incubated at 28 ±0.5 °C for 72 hr and bacterial inoculum with a minimum concentration of 0.25 OD_{600} nm (10^8 CFU/ml) was used for pathogenicity test. To check the pathogenicity of the isolate's protocols reported by Sharma *et al.,* (2017) was followed.

Results and Discussion

Morphological characters of pathogen

The morphological characters like its fuscan development, unique dark brown fuscan pigmentation was observed in *Xap* inoculated NGA medium. Its intensity increased after 7 days of incubation. Production of pigmentation is a unique character of *Xap*, helps in differentiating with other non-pathogenic bacteria. Colonies on NGA plates are circular, convex, mucoid, shiny and yellow were observed (Figure 1).

Molecular identification of pathogen

The molecular identification of the pathogen was established using a primer set, KKM-5 Forward and KKM-6 Reverse primers amplify gyraseB (GyrB) gene designed by Mondal *et al.,* (2012). The primer set gave amplicon size of 491 bp, which is specific to only *X. axonopodis pv. punicae* (Figure 2).

Testing the pathogenicity of pathogen

Six month oldBhagwa plants raised in greenhouse conditions (28 ±0.5°C, 60-70% relative humidity) were used for pathogenicity test.

Preparation of bacterial inoculum

Bacterial inoculum was prepared by inoculating the pure and single colony of *Xap* in NGA media, incubated at 28 ±0.5 °C for 72 hr and bacterial inoculum with a minimum concentration of 0.25 OD_{600} nm (10^8 CFU/ml) was used for pathogenicity test (Plate 10). Symptoms were observed 9 days after inoculation. Initially plants shown water soaked legions on the lower leaf surface and later turned into dark brown spots surrounded by yellow hallo on leaf. From these inoculated leaves pathogen was re-isolated and compared with original culture and also confirmed using specific primers.

Phenotype based identification of *Xanthomonas axonopodis*pv. *punicae*

The morphological characters like its fuscan Development, Unique dark brown fuscan pigmentation was observed in *Xap* inoculated NGA medium. Its intensity increases after seven days of incubation. Production of pigmentation is a unique character of *Xap*, helps in differentiating with other non-pathogenic bacteria. Colonies on NGA plates are circular, convex, mucoid, shiny and yellow were observed. Results of both phenotype tests were in conformity with earlier report by Mondal and Kumar (2011) and Sharma *et al.,* (2017).

Molecular identification of *Xanthomonas axonopodis* pv. *punicae* using specific primers

To identify pathogen a primer set, KKM-5 Forward and KKM-6 Reverse designed by Mondal *et al.,* (2012) were used for molecular detection of pathogen.

The primer set gave product size of 491 bp, which is specific to only *X. axonopodis*pv. *punicae (Xap)*. These results are in accordance with Mondal *et al.,* (2012).

Pathogenicity test

Four month oldBhagwa plants raised in greenhouse conditions (28 ±0.5 °C, 60-70% relative humidity) were used for pathogenicity tests. Preparation of bacterial inoculum: Bacterial inoculum was prepared by inoculating the pure and single colony of *Xap* in NGA media, incubated at 28 ±0.5 °C for 72 hr and bacterial inoculum with a minimum concentration of 0.25 OD_{600} nm (10^8 CFU/ml) was used for pathogenicity test. Symptom was observed 9 days after inoculation. Initially,

plants shown water soaked legions on the lower leaf surface and later turned into dark brown spots surrounded by yellow hallo on leaf.

From these inoculated leaves pathogen was re-isolated and compared with original culture and also confirmed using specific primers.

The symptoms observed during pathogenicity and their confirmation with phenotypic expression is matching with earlier studies as reported by many workers. Hingorani and Mehta (1952) isolated the bacterial pathogen from infected pomegranate leaves and proved pathogenicity.

Infection was readily seen by them on tender leaves of the artificially inoculated plants in seven to ten days with congenial greenhouse conditions. Isolation and pathogenicity studies were also carried out in a similar fashion by Kanwar (1976).

He has observed the symptoms within four to seven days on injured portions and it took eight to twelve days to get the symptoms on uninjured parts.

A primer set, KKM-5 Forward and KKM-6 yielded the product size of 491 bp, which is specific to only *X. axonopodis*pv. *punicae*(*Xap*). Morphological characters like circular, convex, mucoid, shiny and yellow colonies on NGA media were observed.

The symptoms observed during pathogenicity and their confirmation with phenotypic expression is matching with earlier studies as reported by many workers.

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