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Research Article

DEVELOPMENT OF POLYMERASE CHAIN REACTION BASED ASSAY FOR DIAGNOSIS OF BACTERIAL BLIGHT OF POMEGRANATE

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ABSTRACT

Pomegranate (*Punica granatum* L.) diseases often caused by a range of fungi and bacteria, pose direct significant financial, nutritional and postharvest loses along the value chain. A major bacterial disease of pomegranate is bacterial blight caused by *Xanthomonas axanopodis* pv. *punicae*. The problem with traditional diagnosis is that, in most cases, farmers are visually looking for infection spots, especially on the leaf and fruit of the Pomegranate plant, but the infection has already spread very badly before the visual symptoms appear. In the present study, we standardized a rapid PCR based test for the detection of bacterial blight disease. The plant samples, an infected fruit and an infected leaf, will be taken and homogenised for DNA extraction using the CTAB method. The isolated DNA will be then subjected to PCR amplification. *Xanthomonas axonopodis* pv. *punicae* genus-specific primers were used to amplify the gyrB gene product of 491 base pairs. The PCR product was loaded onto a gel electrophoresis system after successful amplification to obtain visible bands of DNA based on their charge to mass ratio. This test will be cost effective and will assist growers in controlling the disease at its earliest stages, minimising yield losses and increasing productivity while maintaining fruit quality.

Keywords: Bacterial Blight, Pomegranate, PCR, Diagnostics, Plant health.

1. INTRODUCTION

One of the favorite table fruits of the tropical and subtropical regions, Pomegranate (*Punica granatum* L.) [1] belongs to the Lythraceae family. The fruit crop is native to Iran and the Himalayas in northern India and has been grown since ancient times in all Mediterranean countries, such as Spain, Morocco, Egypt, Iran, Afghanistan and Baluchistan. It is widely cultivated in India, Malaya, Myanmar, China, Japan, USA (California), and the drier portion of Southeast Asia [2]. A disease outbreak occurred in new pomegranate orchards in South Africa's Western Cape and Limpopo provinces in the spring of 2007 [3], leading to significant crop losses. Branch, stem and nodal tumors, leaf spots and fruit blemishes were the signs. Trees died on a branch or trunk above the parts which were girded by the canker. This is the first bacterial blight research on pomegranate in South Africa and the purpose of this analysis was to isolate and classify the causal organism.

The Pomegranate is an increasingly valuable fruit crop that is widely grown in Turkey [4], since spring 2011, typical bacterial blight symptoms have been observed in pomegranate orchards located in the province of Antalya. On yeast dextrose chalk agar, the pathogen was isolated from leaf spots on naturally infected plants exhibiting typical symptoms. There were consistently isolated bright yellow bacterial colonies. Gram negative bacterial strains have been described and their pathogenic ability on 2-year-old pomegranate plants has been checked. This is the first report of bacterial blight of pomegranate in Turkey and a high incidence of bacterial blight of pomegranate has already been recorded in India, Pakistan and South Africa. In the Indian states of Maharashtra, Karnataka and Andhra Pradesh, bacterial pomegranate blight has become the biggest threat to pomegranate cultivation [5]. All the above ground parts of the plant, including fruits, are attacked by the pathogen, leading to a substantial decrease in fruit quality and market value.

Under epidemic conditions, bacterial blight resulted in yield losses of 60-80 percent in Karnataka to 80 percent in some orchards in Maharashtra. In Karnataka, bacterial blight resulted in yield losses of Rs 200 million during 2007-08, as production fell dramatically from 1.18 million tons in 2003-04 to just 10,000 tons in 2007-08

over a four-year period, when, as in Maharashtra, blight damaged pomegranate cultivation over more than 30,000 ha, resulting in losses of Rs 10,000 million during 2006-07.

In the major pomegranate growing regions of Western Maharashtra, field surveys were conducted, revealing the high prevalence of bacterial blight incidence in the districts of Solapur, Sangli and Nashik. From the highly infected plant materials collected during the field survey, four distinct isolates of this pathogen were extracted. On the basis of the pathogen's morphological, bio-chemical, physiological, hypersensitive and pathogenicity assessments, it was identified as *Xanthomonas axonopodis* pv. *punicae* [6]

Among the five pathogen isolates, which were made from symptomatic samples collected from five different locations belonging to the Ahmednagar district of Maharashtra state, heterogeneity was studied by using 10 RAPD primers. A total of 26 scorable bands with an average of 3.7 bands per primer are fabricated from 7 primers. 21 bands were found to be polymorphic out of 26 bands and the degree of polymorphism was 80.76 percent. These findings show a high degree of genomic variability even within the same geographical regions among the isolates [7].

At a temperature range of 9.0- 43.0°C and relative humidity of 30.0 to 80.0 percent, the disease remained prevalent throughout the year. Its magnitude, however, varied according to the season. During summer and rainy season, the severity of the blight was greater (48,9% of orchards) than during fall (10.5 percent of orchards). The high infection rate (0.21/unit/day) against autumn (0.08/unit/day) demonstrated a rapid increase in blight during the rainy season. Integrated disease management activities involving disease-free planting materials, rainy season crop avoidance, sanitation steps and antibiotic sprays at 15-day intervals resulted in successful Control of blight and higher yields of good quality fruit [8].

Disease-free planting materials are needed to limit the spread of the pathogen. There is a need of a very robust and accurate PCR-based detection system to ensure the presence of pathogens in suspected planting (or cutting) materials [9]. The *gyrB* gene has been shown to be a successful candidate for distinguishing bacterial plant pathogens pathovars including the genus *Xanthomonas*. The *gyrB* gene, particularly its C-terminus variable region, was targeted while developing a PCR-based bacterial blight pathogen detection technique. A template DNA extraction protocol from plant tissue was subsequently optimized and validated. For PCR amplification of 491 bp from the *gyrB* gene, a primer set was synthesized, namely KKM5 and KKM6. In all forms of infected pomegranate samples, including leaves, fruits and stems, the method developed could detect pathogenic microorganism.

Plant diseases have become an issue in agriculture since they lead to losses in production, along with the quality of the agricultural product being affected in certain circumstances [10]. It is important to detect the infection in latent stages rather than in symptomatic stages to reduce economic losses incurred by the bacterial blight. Various microbiological approaches are also available for the diagnosis of bacterial blight, but they are also very time consuming and costly. Our objective is to develop a rapid and reliable PCR-based disease diagnosis process, as PCR is one of the most responsive and specific tests used for genomic detection of various microorganisms. PCR is reasonably easy to understand and use, is highly sensitive and easily generates outcomes producing millions to billion copies of target gene. qRT-PCR offers the same benefits as the PCR, with the additional benefit of quantifying the synthesized product.

2. MATERIAL AND METHODS

2.1. Sample Collection and Transportation

The infected and symptomatic pomegranate plants were identified on the basis of the images and literature published in the articles referred to, and also on the basis of the farmers' experience. These plant species have not been classified as endangered and the collection sites for this study have not been found within a national park or any other restricted forest area. With the permission of the farm owners, all the samples were obtained from two separate farms. Suspected samples were collected from five different fields in Pandharpur Taluka, Solapur District, Maharashtra, in separate bags in accordance with the standard operating procedure. The samples would be getting transported to the MGM Campus Central Research Laboratory, Navi Mumbai, within two days after collection for analysis purposes using appropriate storage conditions in a closed container.

2.2. Isolation of microorganism

Microbial growth requires solid culture media Containing nutrients and physical growth parameters and helps to isolate microorganism and evaluate the isolate's colony characteristics. The isolation of pathogen will be done in two subculture levels.

2.3. Biochemical tests

Well-separated fluid colonies of bacterial isolates will be subjected to biochemical tests such as KOH solubility, Catalase test, Oxidase test and Urease test. The bacterial culture will also be tested for Citrate utilization, H_2S and Indole production. As the pathogen posses a single polar flagellum, it will also be tested for its motility using Mannitol motility test [12].

2.4. Molecular characterization

2.4.1. Genomic DNA isolation

Following confirmation of biochemical characteristics, the next step is to isolate genomic DNA in order to identify the suspected microorganism at the genetic level. This DNA will be used to amplify its primer-specific segment using PCR technique. The presence of a rigid cell wall surrounding plant cells makes DNA extraction from plant tissues difficult. The CTAB method can be applied to both freeze-dried and fresh plant samples [11].

2.4.2. Polymerase Chain Reaction

Xanthomonas axonopodis pv. *punicae* genus-specific primers *i.e.*, KKM-5 Forward 5'-GTTGATGCTGTTCACCAGC G-3', KKM-6 Reverse 5'-CATTCATTTCGCCCAAGC CC-3' were used to amplify the **gyrB gene** product of 491 basepairs. The PCR reaction mixture was prepared under aseptic conditions inside a clean, sterile biosafety cabinet. Aliquots for forward primer and reverse primers were prepared in separate tubes by adding 4 µL of primer to 36 µL of molecular grade water in the ratio of 1:9. To prepare final PCR reaction mixture, for each PCR tube, the reagents were mixed as per the table 1.

After mixing, the prepared PCR tubes were put into the thermal cycler and subjected to following PCR cycles with temperature and time setting at 94°C for 5 minutes (initial denaturation) followed by 30 cycles of amplification consisting of denaturation at 94°C for 30 seconds, annealing at 55°C for 60 seconds and elongation 72°C for 60 seconds followed by a final elongation at 72°C for 3 minutes and cooldown step set at 4°C for 5 minutes [11]. Gel electrophoresis will be used to confirm the successful amplification of the gyrB gene whilst comparing it to a DNA ladder.

Table 1: Reaction mixture for PCR

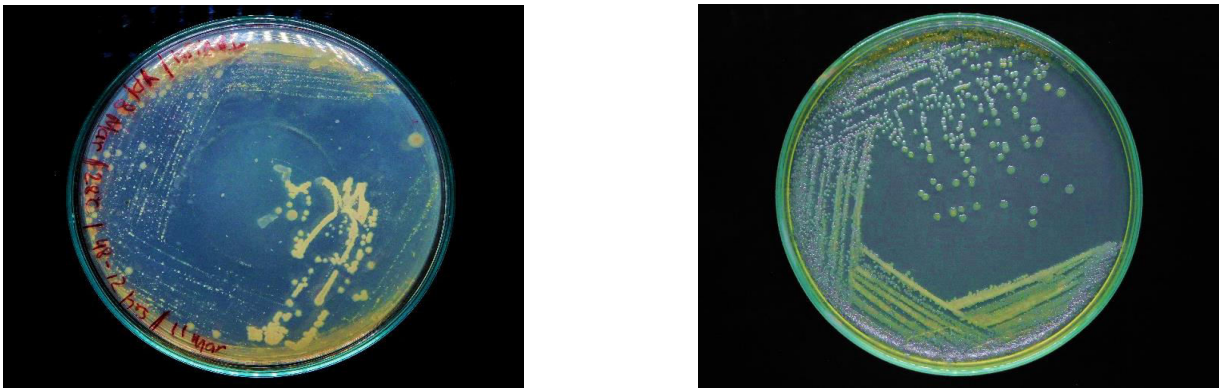
Reagents	Volume (µL)
2X Master mix	12.5
Forward primer	1.0
Reverse primer	1.0
Nuclease free water	5.5
Template DNA	5.0

3. RESULTS

3.1. Isolation of microorganism

The isolation of pathogen was done in two subculture levels. A primary culture tube was made of Peptone water, to which the Pomegranate fruit and leaf extract were inoculated and is incubated for 48 hours for optimum growth of the pathogen. In the first subculture level, the peptone water extract of crushed pomegranate was inoculated on Yeast Peptone agar plate and was incubated undisturbed for 48 to 72 hours at room temperature [9]. Mix growth of two different colonies were seen after 72 hours (Fig. 1a). Gram staining of both the colonies revealed one belong to Gram-positive coccus and other being of Gram-negative bacillus.

The colony with Gram-negative bacillus was selected for further purification. The microorganism colony was purified and classified based on colony characteristics at the second subculture stage (Fig.1b).



a. *first subculture with mixed colonies, from which the big fluidic colonies were selected for* ***b.*** *second subculture of pure colonies of Gram-negative bacillus*

Fig. 1: Yeast Peptone agar isolates

Table 2: Colony characteristics of isolated microorganism

Colony Characteristics	
Size	≈ 1 mm
Shape	Circular, fluid
Colour	Pale yellow
Consistency	Butyrous
Opacity	Opaque
Margin	Entire

Gram staining was performed as per the protocol. Microscopic field produced by 100X oil immersion objective showed slender pink rod-shaped micro-organism (Fig. 2) which was approximately about 1-3 µm long in size hence confirming its identity as a Gram-negative bacillus.

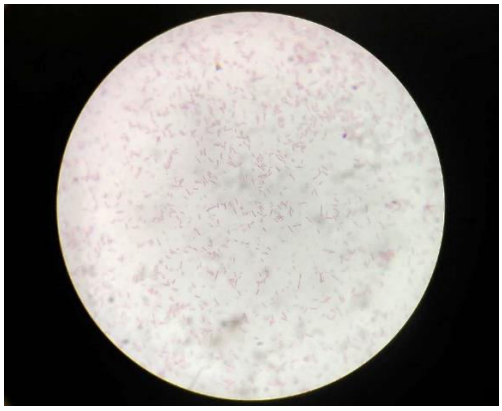


Fig. 2: Representative image of gram staining

3.2. Biochemical tests

The microorganism was assessed on various biochemical parameters, the result of which was compared with what mentioned in Bergey's Manual® of Systematic Bacteriology. The results of biochemical tests were documented in table 3.

Table 3: Biochemical characteristics of isolated bacteria

Biochemical tests	Results
KOH solubility	+
Catalase test	+
Oxidase test	+
Urease test	-
Citrate utilization	+
Indole production	-
H_2S production	+ K/A
Mannitol motility test	+

Legends: + positive, - negative

3.3. Molecular characterization

Amplified PCR products were loaded on 2% agarose gel and were subjected to electrophoresis as per the procedure described in methodology. Two DNA samples from different plant parts, infected fruit and leaves, were amplified using PCR with a Xap specific primer. Lanes 3 and 4 clearly show the successful amplification of 491 bp PCR products, as shown in the Fig. 3. In the negative control samples, no amplification was observed. The above result shows that the PCR assay was successfully standardised for screening Xap from raw infected plant samples.

Fig. 3: Molecular confirmation of *Xanthomonas axanopodis* pv. *punicae* by using gyrB genus specific primer

4. CONCLUSION

The results obtained showed the presence of *Xanthomonas axanopodis* pv. *punicae* from the selected infected pomegranate samples. A series of microbiological and biochemical tests confirmed its presence. The plant samples, an infected fruit and an

infected leaf, were selected and homogenised for DNA extraction using the CTAB method. The isolated DNA was then subjected to PCR amplification. *Xanthomonas axonopodis* pv. *punicae* genus-specific primers i.e., KKM-5 Forward 5'-GTTGATGCTGTTCACCAGCG-3', KKM-6 Reverse 5'-CATTCATTTCGCCCAAGCCC-3' were used to amplify the gyrB gene product of 491 base pairs. The PCR product was loaded onto a gel electrophoresis system after successful amplification to obtain visible bands of DNA based on their charge to mass ratio. The results indicated a successful amplification of the pathogen's gyrB gene of 491 base pairs when compared to a standard 50 base pair DNA ladder.

The entire process was standardised multiple times to reduce turnaround time, and the process, from receiving samples to interpreting results, took no more than 4 hours. During multiple tests, it was discovered that some of the asymptomatic leaf samples contained pathogens, demonstrating the sensitivity of this assay, which can detect pathogens even before symptoms appear to naked eyes. It is possible to conclude that a rapid polymerase chain reaction-based assay for early detection of bacterial blight in pomegranates has been successfully standardised. This test will be cost effective and will assist growers in controlling the disease at its earliest stages, minimising yield losses and increasing productivity while maintaining fruit quality.

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