

Identification and characterization of *Xanthomonas* spp. affecting fruit crops cultivated in Assam, India

ABSTRACT

The present study was carried out to identify the bacterial pathogens affecting different fruit crops and to consign them in proper taxonomic position following different morphological, biochemical and molecular protocols. Diseased samples exhibiting typical symptoms on pomegranate (*Punicagranatum*), mango (*Mangifera indica*), peach (*Prunus persica*), and plum (*Prunus domestica*) were gathered from two locations in Assam (Jorhat and Sonitpur). Through morphological, biochemical, and molecular characterization, the bacterial isolates were identified within the genus *Xanthomonas*, displaying a broad host range encompassing pomegranate, mango, peach, and plum. Phylogenetic analysis of 16S rRNA gene sequences indicated the isolates as *Xanthomonas citri* pv. *mangiferae indicae* (causing bacterial canker in mango), *Xanthomonas axonopodis* pv. *punicae* (responsible for bacterial blight in pomegranate), and *Xanthomonas arboricola* pv. *pruni* (associated with bacterial leaf spot in stone fruits). The findings underscore the importance of *Xanthomonas* in impacting diverse fruit crops, highlighting potential threats to their production.

Keywords: Bacterial diseases, Fruit crops, *Xanthomonas* spp., Morphological and cultural, Biochemical and molecular characterization

Introduction

Cultivation of fruit crops contributes to the health, happiness and prosperity of the people. The standard of living of the people is often judged by production and consumption of fruits per capita. India, as the second-largest global producer of fruits, leads in the cultivation of various fruits, including mango, banana, sapota, pomegranate, and aonla. According to the latest data from the National Horticulture Database (3rd Advance Estimates) released by the National Horticulture Board, India achieved a remarkable output of 107.24 million metric tonnes of fruits and 204.84 million metric tonnes of vegetables during the 2021-22 period. The extensive land devoted to fruit cultivation spans 7.05 million hectares, while vegetables are grown across 11.35 million hectares. The state of Assam, situated in the Northeastern region of India, boasts diverse climates and soils conducive to the cultivation of numerous horticultural crops. These include fruits, vegetables, spices, potatoes, tropical tuber crops, mushrooms, ornamental, medicinal and aromatic plants, as well as plantation crops. Among the

major fruit crops cultivated in the state are banana, pineapple, papaya, Assam lemon, orange, guava, litchi, jackfruit, and mango. This diverse cultivation not only enhances the agricultural landscape of Assam but also contributes significantly to the overall fruit production in India.

There is tremendous potential for large-scale expansion of fruit crops in Assam, which could significantly impact the state's economy. Bacterial pathogens causing plant diseases pose major constraints on crop production, leading to significant annual losses worldwide (Sundin et al., 2016). Despite the severe losses inflicted by bacterial diseases on various fruit crops in different districts of Assam, systematic research for the identification, characterization, and management of these bacterial pathogens is lacking in the region.

The identification and characterization of these pathogens are crucial for understanding specific disease problems and implementing effective management strategies. Unfortunately, comprehensive information regarding their complete characterization is currently unavailable. The present study aims to fill this gap by isolating, identifying, and characterizing bacterial pathogens associated with diseases in fruit crops.

Materials and Methods

Sample collection and isolation of the pathogens: Different parts of the plants like leaves and stem showing characteristic symptoms of bacterial infection were collected from two distantly located (about 235 km) places of Assam namely Jorhat and Sonitpur. The collected plant samples (*i.e.*, mango, pomegranate, peach and plum) were brought to the Department of Plant Pathology, AAU, Jorhat for further studies. Observation of symptoms of blight on pomegranate (*Punicagranatum*) was recorded on both leaves and fruits. Brown necrotic spots surrounded by yellow halo were observed on the leaves while brown to black raised oily spots were seen on the fruits (Fig.1a). The symptoms like brown angular to irregular lesions surrounded by yellow halo on diseased mango (*Mangifera indica*) leaves were seen (Fig.1b). The symptoms on peach (*Prunus persica*) (Fig.1c) and plum (*Prunus domestica*) were seen as brown or black spots surrounded with yellow halo while some leaves showed a typical shot-hole appearance. On the plum fruit small rough pit-like lesions were found (Fig.1d). The diseased tissues with a little portion of healthy tissues were cut into small pieces and surface sterilized with 0.1 per cent sodium hypochlorite solution for 60 sec. It was washed with sterilized distilled water for three times to remove traces of sodium hypochlorite solution. The cut pieces were placed onto sterile Petri plates containing Nutrient Agar (NA) medium. The plates were incubated for 2–3 days at 28°C. The pure culture of the isolates were obtained in nutrient agar medium as well as in yeast glucose

chalk agar (YGCA) medium and kept in the incubator for 48 h at 28±2°C. The cultures were maintained in the refrigerator at 4°C which served as a stock culture for further studies. The pathogens isolated were designated as RC4 (mango), RC5 (pomegranate), RC6 (peach) and RC7 (plum) for convenience in study (Fig.2).

Pathogenicity test: Detached leaf inoculation technique (Randhawa and Civerolo,1985) was followed for proving pathogenicity of the isolates obtained from the diseased samples. Three middle aged leaves were selected and detached from the plants. They were washed well in tap water, swabbed with 70 per cent ethanol and allowed to dry. Then injuries were made at several points by pricking with sterilized needle laid with 10⁹ cfu/ml inoculums of the isolates and also smeared on both sides with culture soaked sterilized cotton swab. The leaves were kept in plates which were lined with sterilized moist filter paper to maintain humidity and incubated at 30°C. One plate was taken as control and the leaves were inoculated with sterile water. The disease symptoms were recorded at different time interval.

Cultural, morphological and biochemical characterization of the isolates: Morphological and cultural characteristics, encompassing attributes like shape, size, colony shape, colony color, colony elevation, gram staining, KOH test, pigment production, oxygen requirement, and growth on different media, were thoroughly examined. The morphological features of the isolates were investigated using the Carl Zeiss Sigma Field Emission Scanning Electron Microscope. Additionally, the bacterial isolates underwent various biochemical tests, including citrate utilization, starch hydrolysis, lysine utilization, urease production, phenylalanine deamination, nitrate reduction, H₂S production, and carbohydrate utilization. These biochemical tests were performed using the KB002 and KB009 biochemical test kit from Himedia, India.

Molecular characterization and phylogenetic analysis: Genomic DNA of the bacterial isolates was extracted by following the modified method of Cardinal *et al.* (1997). The extracted genomic DNA was amplified using universal primer pairs universal primers U16SF (5-AGAGTTTGATCMTGGCTCAG-3) and U16SR (5-TACGGYTACCTTGTTACGACTT-3) (Goswami *et al.*, 2017). PCR assay was performed in 10 µl reaction mixture containing 0.5 µl of template DNA; 5 µl Emerald Amp GT PCR Master Mix (2X Premix) (Takara); 0.5 µl Forward primer (10 pmol/µl); 0.5 µl Reverse primer (10 pmol/µl) and 3.5 µl sterile distilled water. The PCR was carried out at an initial denaturation of 94°C for 3 min, followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 55°C for 1 min, extension at 72°C for 1.30 min, with the final extension at 72°C for 7 min. PCR products were separated in 1.5 per cent agarose gel and results were observed in Gel Doc. The PCR products of the samples were sent to Bioserve Biotechnologies Private Limited, Hyderabad respectively for 16S rRNA sequencing. The 16S

rDNA sequence reads obtained after sequencing were assembled into contig using CodonCode Aligner (CodonCode Corporation, USA). Further, sequence similarity tool BLAST was employed to find the similarity of the sequences with known 16S rDNA sequences available in the NCBI GenBank databases. Phylogenetic analysis of the 16S rRNA sequences were carried out using the neighbor-joining tree by MEGA-X (Molecular Evolutionary Genetics Analysis) software with Kimura-2 parameter model method with 500-step bootstrap (Kumar *et al.* 2018).

Results and Discussions

Results

The survey was conducted in two distinct locations in Assam, India, where samples suspected to be infected with bacterial pathogens causing diseases were collected. The disease symptoms induced by the bacterial genus *Xanthomonas*, observed on the collected samples, were briefly described in the preceding section. Morphological and cultural studies revealed that the bacterial isolates possessed rod-shaped cells. The bacterial cells were gram-negative, aerobic, rod-shaped, with sizes ranging from 0.3 to 1.8 μm (Fig. 4), and were non-spore formers. All isolates developed colonies that were smooth, round, entire, deep yellow, convex, mucoid, glistening, opaque, and had colony sizes ranging from 2 to 3 mm in diameter. The observed results were consistent with those characteristic of the genus *Xanthomonas* (Table 1). The findings regarding various biochemical characteristics of the bacterial isolates are presented in Table 2. The results indicated that isolates from mango, pomegranate, peach, and plum exhibited positive results for the KOH test, citrate utilization, gelatin liquefaction, oxidase, indole production, levan production, and arginine dihydrolase. Additionally, all isolates showed positive results for the catalase test, indicating the aerobic nature of the bacteria. However, isolates from peach and plum demonstrated a positive result for the nitrate reduction test, evidenced by the formation of gas bubbles in the Durham tubes of the broth, while isolates from mango and pomegranate showed negative results. Furthermore, isolates from peach and plum did not hydrolyze starch, whereas isolates from mango and pomegranate exhibited a positive result for starch hydrolysis. Results for different carbohydrate utilization tests conducted using the biochemical test kit (KB002 and KB009; Himedia, India) are also presented in Table 2. In the pathogenicity test, typical symptoms were observed on the inoculated hosts. Mango leaves exhibited water-soaked lesions that later transformed into brown to black angular leaf spots after three days of inoculation. The pathogen was re-isolated from the observed symptoms on nutrient agar, displaying the same growth characteristics as observed earlier. On pomegranate leaves, symptoms appeared as small water-soaked lesions three days after inoculation, turning into brown to black-colored lesions after six days. The re-isolated pathogen was found identical to the original culture. Peach leaves displayed water-soaked lesions

that later developed necrotic brown spots, expanding to produce a shot-hole. Re-isolation of bacterial strains from the diseased peach leaves confirmed their identity with the original isolates. Plum leaves exhibited symptoms three days after inoculation as small water-soaked lesions, which later turned brown to black in color. Re-isolation of the pathogen from the symptomatic leaves showed similarity with the original culture (Fig. 3). However, no symptoms developed in the control cases.

Sequencing of 16S rRNA was performed to identify the bacterial isolates. The nucleic acid of the isolates subjected to agarose gel (1.5%) electrophoresis yielded one distinct amplicon of 1200-1400 bp in size (Fig. 5). The nucleotide BLAST search revealed the highest nucleotide similarity of the bacterial isolates with 10 different strains of the respective genera. Sequence comparison of the 16S rRNA gene of the isolate from mango with GenBank entries further confirmed the identity as the similarity percentage was 100% to that of *Xanthomonas citri* pv. *mangiferae indicae*. The BLAST results showed highest nucleotide similarity (100%) of the pomegranate bacterial isolate with *Xanthomonas axonopodis* pv. *punicae*. Similarly, the BLAST results for isolates from peach and plum exhibited highest homology with the strains of *Xanthomonas arboricola* pv. *pruni* with 100% similarity (Fig. 6). Further, the phylogenetic tree was constructed using 16S rRNA sequences of the isolates along with the sequences retrieved from NCBI GenBank databases. The results from the phylogenetic analysis indicated that each bacterial isolate was clustered to its corresponding strain from GenBank based on their sequence homology which was reflected by the bootstrap value in the node. These results also indicated the isolates to be *X. citri* pv. *mangiferae indicae*, *X. axonopodis* pv. *punicae*, and *X. arboricola* pv. *pruni* causing bacterial canker in mango, bacterial blight disease in pomegranate and bacterial leaf spot of peach and plum, respectively.

Discussions

The agri-based Indian economy is heavily depended on production and export of fruit crops and thus has helped in enhancing the economic status of the country. Diseases of bacterial origin cause severe loss to these fruit crops in different states of India. However, among the bacterial diseases of plants, diseases caused by the genus *Xanthomonas* is of great economic importance because of its wide host range (Sharma *et al.*, 2014). In our study, the nature of the symptoms caused by the genus *Xanthomonas* in pomegranate, mango, peach and plum were similar to what has been reported for by Jami *et al.* (2005), Pruvost *et al.* (2011), Bora and Katakai (2014), Jadalla and Saad (2018) and Robe *et al.* (2018). All the biochemical characters under the present study were co-related with the characters for all four isolated pathogens as described in the Bergey's Manual of Systematic Bacteriology

(Buchanan and Gibbons, 1984). The identification of the bacterial isolates in the present investigation with respect to symptoms, morphological, biochemical characters and pathological study are in agreement with the previous descriptions (Hingorani and Mehta 1952, Mondal and Singh 2009; Icozet *et al.*, 2014, Ofoeet *al.* 2016, Chowdappaet *al.* 2018). The study of these characteristics played an important role in identifying the genus as *Xanthomonas*. Classical methods like biochemical and carbohydrate utilization tests were also used for identification and differentiation of bacteria; however, these tests cannot distinguish among the closely related species (Goswami *et al.* 2017). The 16S rDNA sequencing has been widely used as a reliable tool for identification and establishing phylogenetic relationships among bacteria (Borsodiet *al.*, 2010). Various studies also indicated 16S rDNA sequence analysis as an authenticated technique to review bacterial isolates at species level (Garrity and Holt 2001, Alamet *al.* 2011). Hence the identity of the bacterial pathogen was confirmed in present study as *Xanthomonas* spp. based on the nucleotide sequencing. In addition to the identification of bacterial pathogens, confirmation of the disease could be done by matching the characteristics described by Pruvostet *al.* (2011) and Ofoeet *al.* (2016) for *X.citripty. mangiferaeindicae* causing bacterial canker in mango. In case of bacterial blight of pomegranate, earlier reports (Chowdappaet *al.* 2018, Sharma *et al.* 2017) helped in ascertaining the disease caused by *X. axonopodispv. punicae*. Previously, the occurrence of bacterial leaf spot of peach caused by *X. arboricolapv. pruni* was reported from other countries like Iran (Jami *et al.* 2005) and China (Robe *et al.* 2017). On the other hand, Shenet *al.* (2013) reported bacterial leaf spot disease on Japanese plum caused by *X. arboricolapv. pruni* from Taiwan. Ritchie (1995) viewed bacterial spot of stone fruit (BSSF) as the most economically important disease of peach, nectarine, Japanese plum, apricot and almond which motivated to carry out the present investigation on bacterial disease of peach and plum along with two most important fruit crops (cash crops) of India *viz.*, mango and pomegranate. To our knowledge these bacterial diseases were not reported earlier from Assam although Bora and Katakai (2014) first reported bacterial blight disease on pomegranate from this state.

Conclusion

The current study sheds light on the prevalence of bacterial diseases attributed to the genus *Xanthomonas* and underscores its significance in fruit crops. A comprehensive investigation encompassing all fruit crop diseases in the North Eastern Region (NER), including Assam, is imperative to comprehend the biodiversity of these plant pathogenic microflora. This understanding is essential for devising effective management strategies. The favorable climate in the entire North Eastern region, especially Assam, provides ideal conditions for the commercial cultivation of fruit crops. Consequently, there is a pressing need for more extensive studies on bacterial diseases,

coupled with an in-depth exploration of pathogen taxonomy, to harness the full potential of fruit crop cultivation in this region.

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Character	Feature	Mango	Pomegranate	Peach	Plum
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Morphological	Size (μm)	0.3x1.7	0.3x1.8	0.3x1.8	0.4x1.9	
	Shape	Rod	Rod	Rod	Rod	
Colony on NA plate	Colony shape	Circular	Circular	Circular	Circular	
	Surface	Smooth, mucoid, glistening	Smooth, glistening	Smooth, mucoid, glistening	Smooth, mucoid, glistening	
	Edge	Entire	Entire	Entire	Entire	
	Colour	Deep yellow	Yellow	Bright yellow	Yellow	
	Elevation	Convex	Convex	Convex	Convex	
	Opacity	Opaque	Opaque	Opaque	Opaque	
	Size (mm)	2-3	2.5-3	2-2.5	2-3	
Cultural	Gram reaction	(-)	(-)	(-)	(-)	
	Pigment production	YGCA	(+)	(+)	(+)	(+)
		King's B	(-)	(-)	(-)	(-)
	KOH test	(+)	(+)	(+)	(+)	
	Oxygen requirement	Aerobic	Aerobic	Aerobic	Aerobic	
Pathological test	On host crop	(+)	(+)	(+)	(+)	

Table 1 Morphological characters of the *Xanthomonas* spp. isolated from four fruit plants

(+) - positive reaction, (-) - negative reaction

Table 2 Biochemical characters of the *Xanthomonas* spp. isolated from four fruit plants

Tests	Mango	Pomegranate	Peach	Plum	Tests	Mango	Pomegranate	Peach	Plum
KOH	+	+	+	+	Cellobiose	+	-	-	-
Catalase	+	+	+	+	Melezitose	-	-	-	-
Oxidase	+	+	+	+	α -Methyl-D-mannoside	-	-	-	-
Starch hydrolysis	+	+	+	+	Xylitol	-	-	-	-
Levan production	+	+	+	+	ONPG	+	-	-	-
Citrate utilization	+	+	+	+	Esculin hydrolysis	+	+	+	+
Nitrate reduction	-	-	+	+	D-Arabinose	+	-	-	-
Gelatin	+	+	+	+	Sorbose	-	-	-	-
Arginine dihydrolase test	+	+	+	+	Malonate utilization	-	+	+	+
Indole production	+	+	+	+	Inulin	+	-	+	+
Lactose	-	+	+	+	Sodium gluconate	+	-	-	-
Xylose	+	-	-	-	Glycerol	-	-	+	+
Maltose	+	-	+	+	Salicin	+	-	+	+
Fructose	+	-	+	+	Dulcitol	-	-	-	-
Dextrose	+	-	+	+	Inositol	-	-	+	+
Galactose	+	-	+	+	Sorbitol	-	-	+	+
Raffinose	-	-	-	-	Mannitol	+	-	-	-
Trehalose	+	+	-	-	Adonitol	-	+	-	-
Melibiose	-	-	-	-	Arabitol	-	-	-	-
Sucrose	+	+	-	-	Erythritol	-	-	-	-
L-Arabinose	+	-	+	+	α -Methyl-D-glucoside	-	-	-	-
Mannose	+	-	+	+	Ornithine utilization	-	-	+	-
Urease	-	-	-	-	Phenylalanine deamination	+	+	+	+
H ₂ S production	-	-	-	-	Rhamnose	-	-	+	+

(+) - positive reaction, (-) - negative reaction

FIGURES

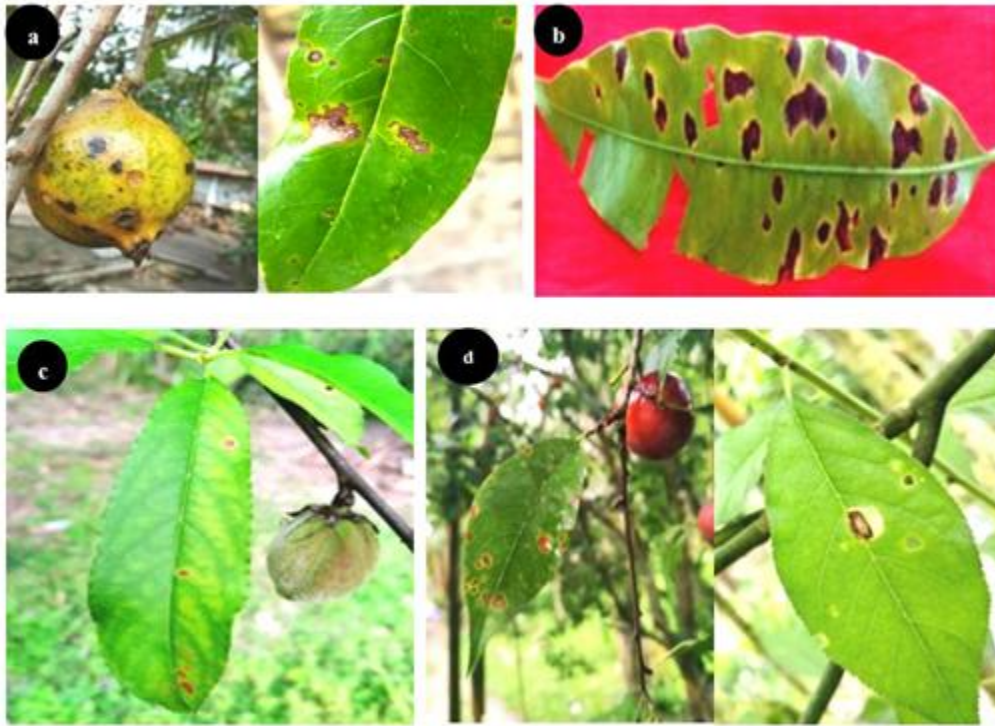


Fig. 1 (a) Brown necrotic spots on diseased Pomegranate (*Punicagranatum*) leaves surrounded by yellow halo; brown to black raised oily spots on fruits. (b) Brown angular to irregular lesions surrounded by yellow halo on diseased mango (*Mangifera indica*) leaves. (c) Small brown spots with yellow halo in diseased Peach (*Prunus persica*) leaves. (d) Small brown spots surrounded by yellow halo in diseased Plum (*Prunus domestica*) leaves; Shot-hole symptoms appearance in the diseased leaves



Fig. 2 Pure culture of the bacterial isolates in nutrient agar (NA) slants. RC4= mango isolate; RC5= pomegranate isolate; RC6= peach isolate and RC7= plum isolate

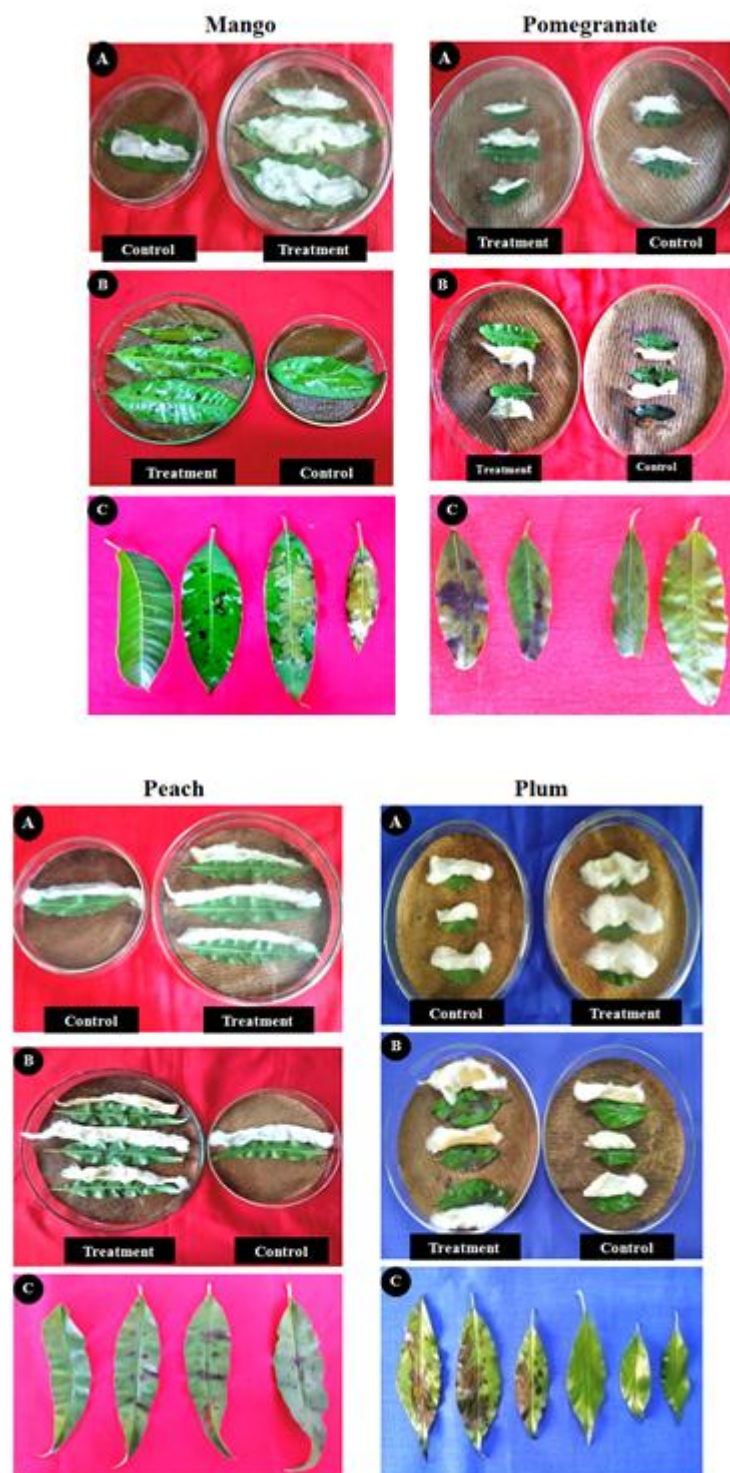


Fig. 3 (A) Inoculation of the fruit plant leaves of mango, pomegranate, peach and plum with the bacterial isolates by detached leaf technique; (B) Appearance of

symptoms on the inoculated leaves; (C) Comparison of healthy and inoculated leaves

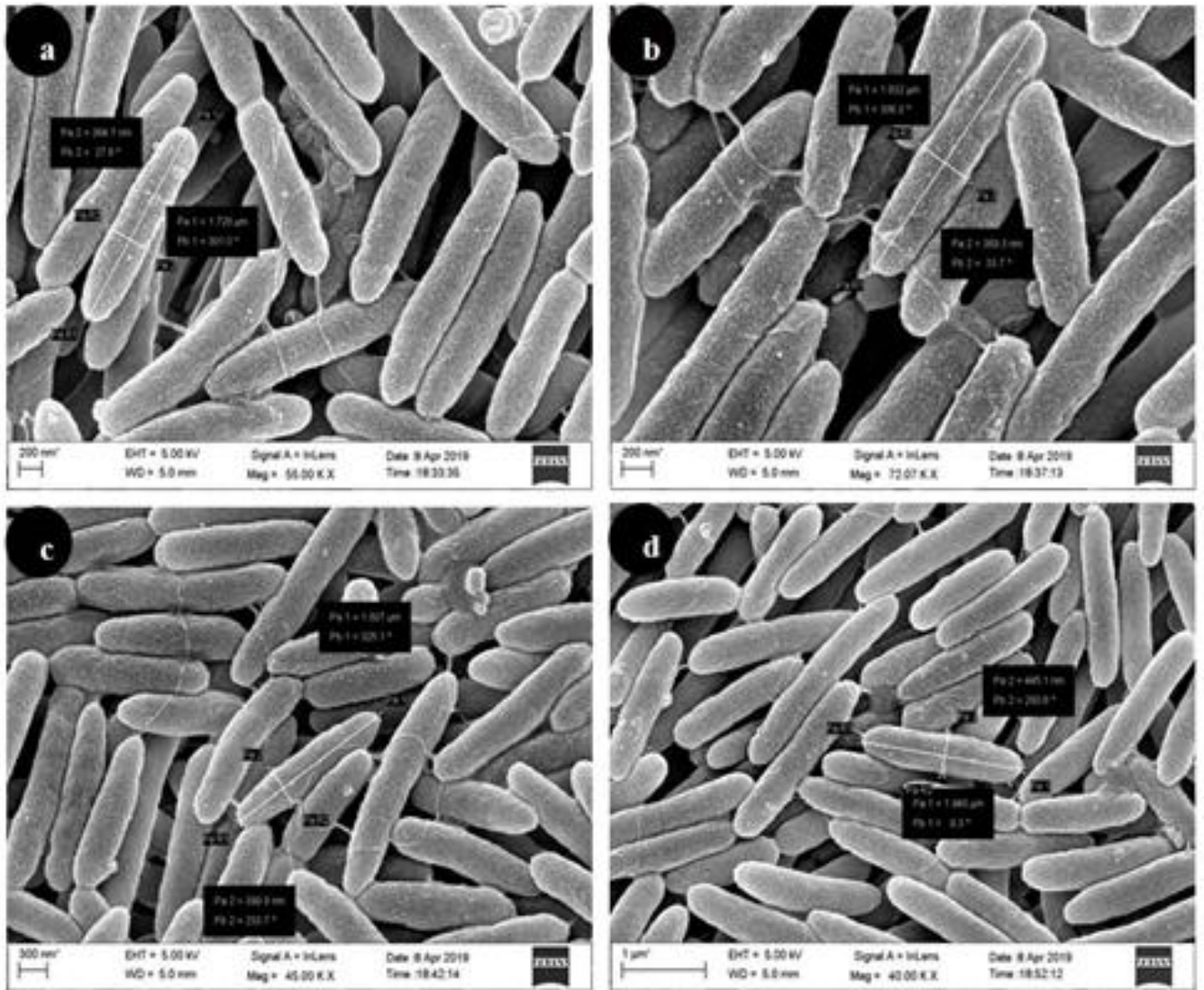


Fig. 4 Scanning electron micrograph of *Xanthomonas* spp. isolated from four fruit plants. (a) *X. citripv. mangiferaeindica* from mango, (b) *X. axonopodispv. punicae* from pomegranate, (c) *X. arboricolapv. prunif* from peach and (d) *X. arboricolapv. prunif* from plum

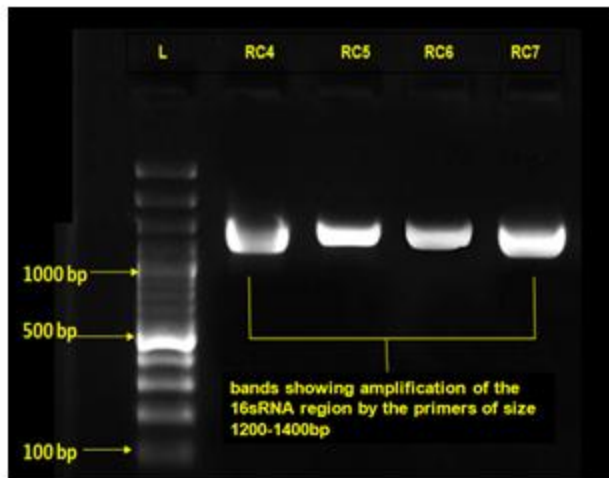


Fig. 5 Agarose gel electrophoresis showing amplification of the DNA products of bacterial pathogen *Xanthomonas* species. L-100bp ladder. RC4 -Mango isolate *X. citri*pv. *mangiferaeindicae*, RC5 - Pomegranate isolate *X. axonopodis*pv. *punicae*, RC6 -Peach isolate *X. arboricola*pv. *pruni*, RC7 -Plum isolate *X. arboricola*pv. *pruni*

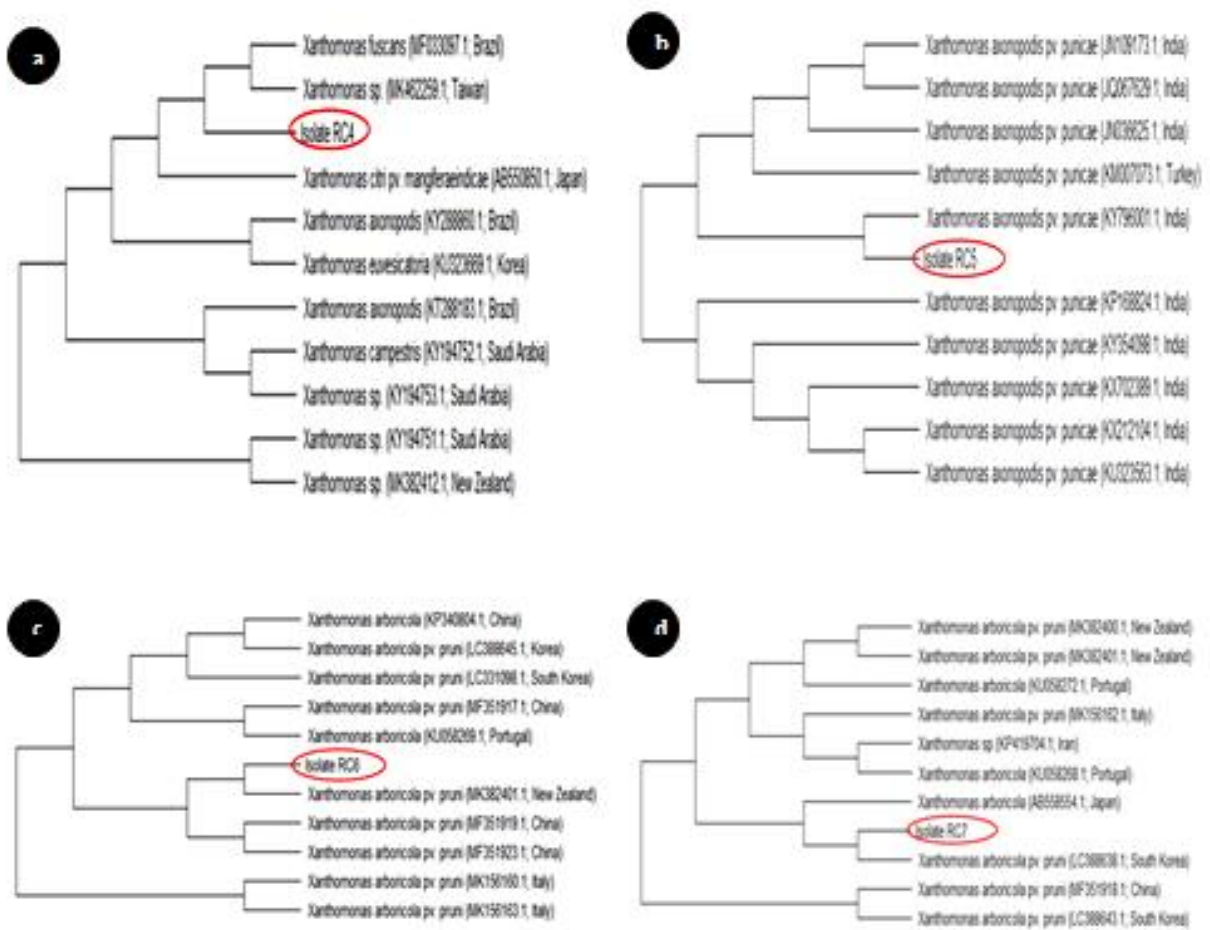


Fig. 6 Phylogenetic tree showing the genetic relationship of the bacterial isolates (*Xanthomonas* spp.) with other strains by using maximum likelihood method with 500 bootstrap replicates.

RC4 -Mango isolate *X. citri*pv. *mangiferaeindicae*, **RC5** -Pomegranate isolate *X. axonopodis*pv. *punicae*,
RC6 -Peach isolate *X. arboricola*pv. *pruni*, **RC7** -Plum isolate *X. arboricola*pv. *pruni*