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

Comment [f1]: Modify the title to make it scientifically catchy.

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. The diseased samples showing typical symptoms on the fruit crops, *viz*., pomegranate (*Punica granatum*), mango (*Mangifera indica*), peach (*Prunus persica*) and plum (*Prunusdomestica*) were collected from different locations of Assam (Jorhat and Sonitpur). On the basis of morphological, biochemical and molecular characterization, the bacterial isolates were identified under the genus *Xanthomonas* with multiple hosts (pomegranate, mango, peach and plum). The phylogenetic analysis of 16S rRNA gene sequences indicated the isolates to be *Xanthomonas citri* pv. *mangiferaeindicae* (bacterial canker in mango), *Xanthomonas axonopodis* pv. *punicae* (bacterial blight ofpomegranate), *Xanthomonas arboricola* pv. *pruni* (bacterial leaf spot of stone fruits).

Comment [f2]: Change it as 'two' or 'two geographically different'

Comment [f3]: Add one last sentence of conclusion.

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

Comment [f4]: Scientifically incorrect; change it as morphological or cultural

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, being the second largest producer of fruits, produces highest quantity of fruits like 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, in the 2021-22 period, India generated 107.24 million metric tonnes of fruits and 204.84 million metric tonnes of vegetables. The land allocated for fruit cultivation encompassed 7.05 million hectares, while vegetables were grown over 11.35 million hectares. The state of Assam situated in the Northeastern region of India has a wide range of climate and soils on which a large number of horticultural crops such as fruits, vegetables, spices, potato and other tropical tuber crops, mushroom, ornamental, medicinal and

aromatic plants and plantation crops can be grown. Major fruit crops of the state are banana, pineapple, papaya, Assam lemon, orange, guava, litchi, jack fruit and mango.

There is a tremendous scope for large scale expansion of fruit crops in Assam which will have considerable impact on the state's economy.Plant diseases caused by bacterial pathogens impose major constraints on crop production and cause significant annual losses worldwide (Sundin *et al*. 2016). Although bacterial diseases cause severe loss to different fruit crops in several districts of Assam, however, no systematic research has been conducted for identification, characterization and management of these diseases in Assam. Identification and characterization might help to understand the specific disease problem and will help in management of the pathogens associated with diseases. However, detail information related to their complete characterization has not been done. The present study was made for isolation, identification and characterization of bacterial pathogens associated with diseases of fruit crops.

Comment [f5]: Needs rephrasing to make it grammatically acceptable

Comment [f6]: Characterization of diseases means?

Comment [f7]: Improve the writing with the help of English language reviewer

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 theleaves 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 (Tuite 1969) 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 10^9 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 such as shape, size, colony shape, colony colour, colony elevation, gram staining, KOH test, pigment production, oxygen requirement and growth on different media were studied. The morphological characters of the isolates were studied using Carl Zeiss Sigma Field Emission Scanning Electron Microscope. The bacterial isolates were subjected to various biochemical tests such as citrate utilization test, starch hydrolysis, lysine utilization, urease production, phenylalanine deamination, nitrate reduction, H_2S production, and carbohydrate utilization tests conducted on the biochemical test kit (KB002 and KB009; 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).

Comment [f8]: Recent reference may be provided if available.

Comment [f9]: Rephrase

Results and Discussions

The survey was carried out in different locations of Assam, India where suspected diseased samples infected with bacterial pathogens were collected. The characteristics disease symptoms caused by the bacterial genus *Xanthomonas* observed on the collected diseased samples were briefly described in the previous section.The symptoms of suspected bacterial blight on leaves and fruits of pomegranate (*P.granatum*) were recorded. Brown necrotic spots surrounded by yellow halo were observed on the leaves while brown to black raised oily spots were seen on the fruits. The symptoms of bacterial canker on mango (*M. indica*) appeared as brown angular to irregular lesions surrounded by yellow halo on diseased leaves. The symptoms on peach (*P. persica*) and plum (*P. 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 wereseen. The morpho-cultural study revealed the bacterial isolates to possess rod shaped cells. The bacterial cells were gram-negative, aerobic, rod-shaped with the size ranging from 0.3-1.8µm (Fig.4) and non-spore former. All isolates developed colonies that are smooth, round, entire, deep yellow, convex, mucoid, glistening, opaque and colony size ranging from 2-3 mm in diameter. The results observed were typical to that of the genus *Xanthomonas*(Table 1).The results obtained on various biochemical characteristics of all the bacterial isolates are presented in (Table 2). The results indicated that all the isolates from mango, pomegranate, peach and plum showed positive result for KOH test, citrate utilization, gelatin liquefaction, oxidase, indole production, levan production as well as arginine dihydrolase. The isolates also showed positive results for catalase test indicating the bacteria to be aerobic. However, the isolates from peach and plum showed positive result for nitrate reduction test by formation of gas bubbles in the Durham tubes of the broth while isolates from mango and pomegranate showed negative results. Also, the isolates from peach and plum did not hydrolyze starch whereas both the isolates from mango and pomegranate showed positive result for starch hydrolysis. In addition, the results for different carbohydrate utilization tests conducted on the biochemical test kit (KB002 and KB009; Himedia, India**)** for all the isolates are presented in the Table 2. In pathogenicity test, typical symptoms were observed on the inoculated hosts. The symptoms on the mango leaves appeared as water-soaked lesion which later became brown to black angular leaf spot after three days of inoculation. The pathogen was re-isolated from the observed symptoms on nutrient agar which exhibited the same growth characteristics as observed earlier. The characteristic symptoms were observed on pomegranate leaves after three days of inoculation as small water-soaked lesions. After six days of inoculation, it turned brown to black coloured lesions. The pathogen was re-isolated and it was found identical with the original culture. The symptoms appeared on peach leaves as water-

Comment [f10]: Needs to be presented by splitting the text into small paragraphs.

Comment [f11]: Different locations? Contradicting.
It is mentioned under 'Materials section' that the samples of diseased plants were collected from two places.

Comment [f12]: Redundant information. Already given under 'Materials' section.
Needs to be deleted either here or in 'Materials' section.

soaked lesions which later developed necrotic brown spots which expanded to produce a shot-hole. The diseased peach leaves were used to re-isolate bacterial strains and were found identical with the original isolates. The symptoms appeared three days after inoculation as small water-soaked lesions on the leaves of plum. The spots later turned brown to black in colour. Re-isolation of the pathogen from the symptomatic leaves showed similarity with the original culture (Fig.3). However, there were no symptoms developed for the control in all the 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 ampliconof 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. *mangiferaeindicae*. 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. *mangiferaeindicae*, *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.

Comment [f13]: Present it as a separate paragraph.

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 Kataki (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

Comment [f14]: Needs rephrasing. What is the correlation?
Identification of bacteria is achieved by following the systematic approaches prescribed in Bergey's manual

descriptions (Hingorani and Mehta 1952, Mondal and Singh 2009; Icoz *et al*., 2014, Ofoe *et al*. 2016, Chowdappa *et 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 (Borsodi *et 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, Alam *et 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 Pruvost *et al*. (2011) and Ofoe *et al*. (2016) for *X.citri* pv. *mangiferaeindicae* causing bacterial canker in mango. In case of bacterial blight of pomegranate, earlier reports (Chowdappa *et al*. 2018, Sharma *et al*. 2017) helped in ascertaining the disease caused by *X. axonopodis* pv. *punicae*. Previously, the occurrence of bacterial leaf spot of peach caused by *X. arboricola* pv. *pruni* was reported from other countries like Iran (Jami *et al*. 2005) and China (Robe *et al*. 2017). On the other hand, Shen *et al*. (2013) reported bacterial leaf spot disease on Japanese plum caused by *X. arboricola* pv. *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.

Comment [f15]: Is there any common risk factor for the infection caused by Xanthomonas spp. on these four fruit crops selected in the present study? Need interpretation.

Conclusion

The present research has thrown some light regarding the occurrence of bacterial diseases caused by the genus *Xanthomonas* and its importance in fruit crops. Detailed study covering almost all the diseases of fruit crops of NER including Assam required to know the biodiversity of these plant pathogenic microflora so that proper management strategy can be adopted. To our knowledge these bacterial diseases were not reported earlier from Assam although Bora and Kataki (2014) first reported bacterial blight disease on pomegranate from this state.

Comment [f16]: Bring it under 'Discussion'.

Future Scope

Comment [f17]: Club it with 'Conclusion'

The entire North Eastern states including Assam endowed with a favourable climate and hold a high potential for commercial cultivation of fruit crops, therefore, more elaborative studies on bacterial diseases along with taxonomy of the pathogens is necessary.

Ethical approval

No animals or human participants were involved during any procedures performed in the study

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Comment [f18]: Needs to be formatted according the "Author guidelines" of the journal

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Character	Feature	Mango	Pomegranate	Peach	Plum

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

Comment [f19]: Characteristics

Tests	Mango	Pomegranate	Peach	Plum	Tests	Mango	Pomegranate	Peach	Plum
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		(+)	(+)	(+)	(+)			

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

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

Comment [f20]: Characteristics

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_2S production	-	-	-	-	Rhamnose	-	-	+	+

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

FIGURES

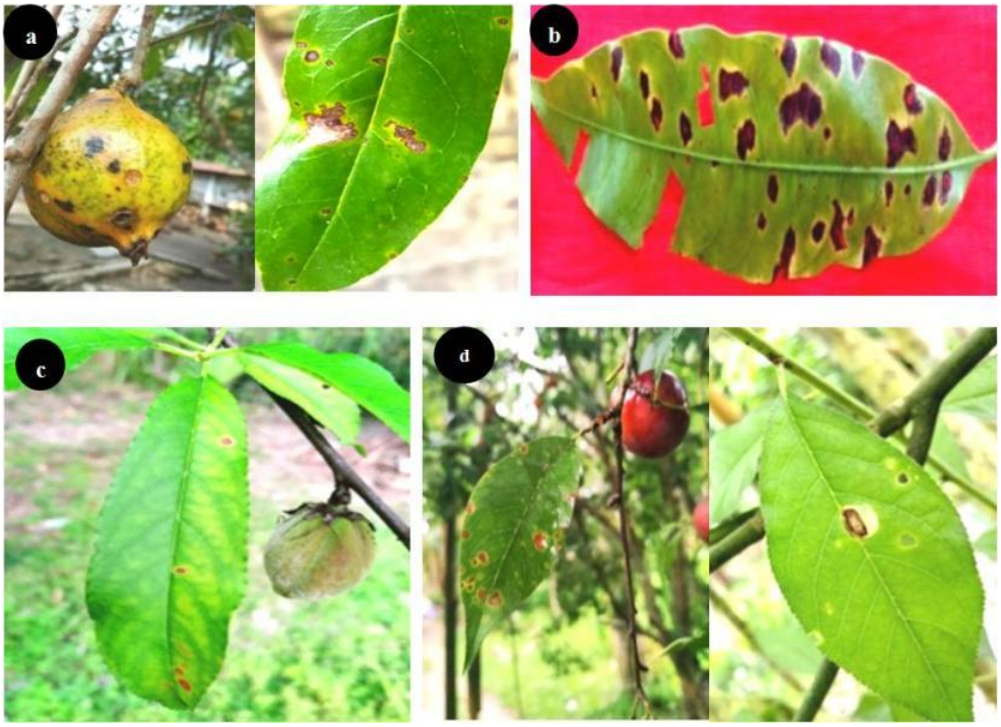

Fig. 1 (a) Brown necrotic spots on diseased Pomegranate (*Punica granatum*) leaves surrounded by yellow halo; brown to black raised oily spots on fruits. (b) Brown angular to irregular lesions surrounded by yellow halo on diseased mango (*Mangifera indica*) leaves. (c) Small brown spots with yellow halo 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

Comment [f21]: Description of disease symptoms is not necessary.
Write the name of the plant and the disease.

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

Comment [f22]: Name of the Bacteria?

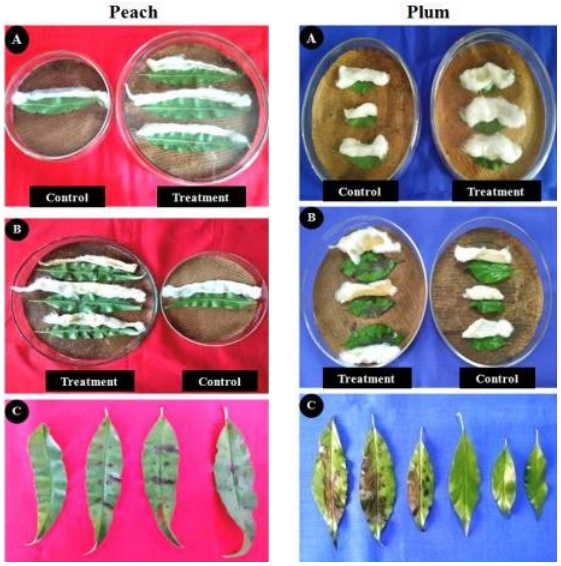

Fig. 3 (A) Inoculation of the fruit plant leaves of mango, pomegranate, peach and plum with the bacterial isolates by detached leaf technique; (B) Appearance of symptoms on the inoculated leaves; (C) Comparison of healthy and inoculated leaves

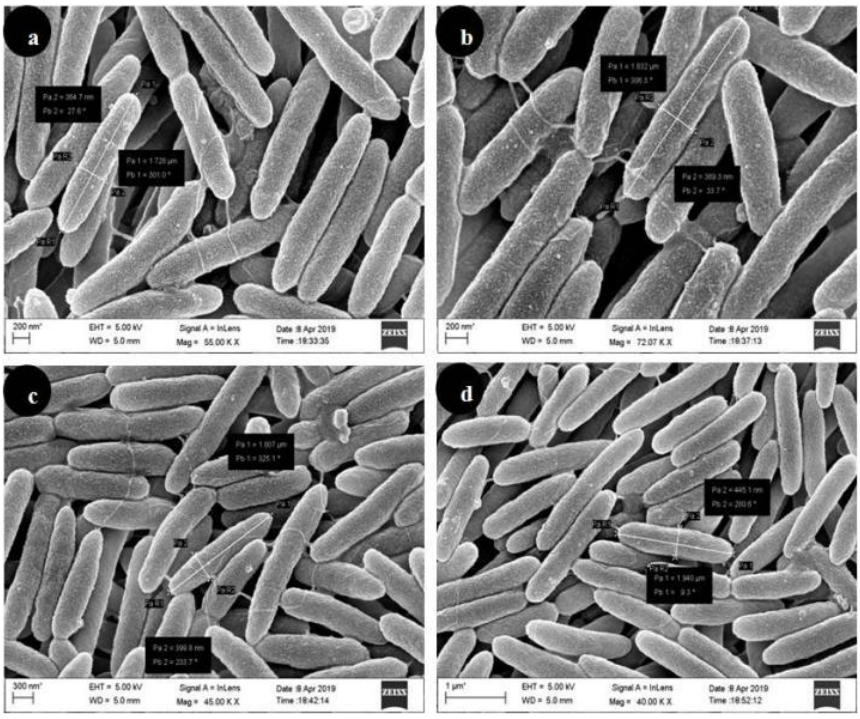

Fig. 4 Scanning electron micrograph of *Xanthomonas* spp. isolated from four fruit plants. (a) *X. citri* pv. *mangiferaeindicae* from mango, (b) *X. axonopodis* pv. *punicae* from pomegranate, (c) *X. arboricola* pv. *pruni* from peach and (d) *X. arboricola* pv. *pruni* from plum

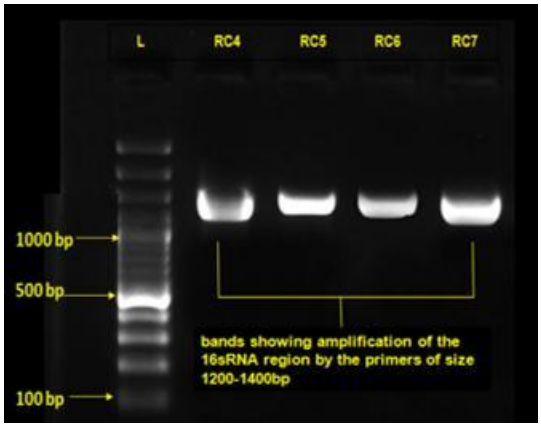

Fig. 5Agarose gel electrophoresis showing amplification of the DNA products of bacterialpathogen *Xanthomonas* species.
L-100bp ladder. RC4 -Mango isolate*X. citri* pv. *mangiferaeindicae*, RC5 -Pomegranate isolate*X. axonopodis* pv. *punicae*, RC6 -Peach isolate*X. arboricola* pv. *pruni*, RC7 -Plum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

Comment [f23]: *Xanthomonas* spp.

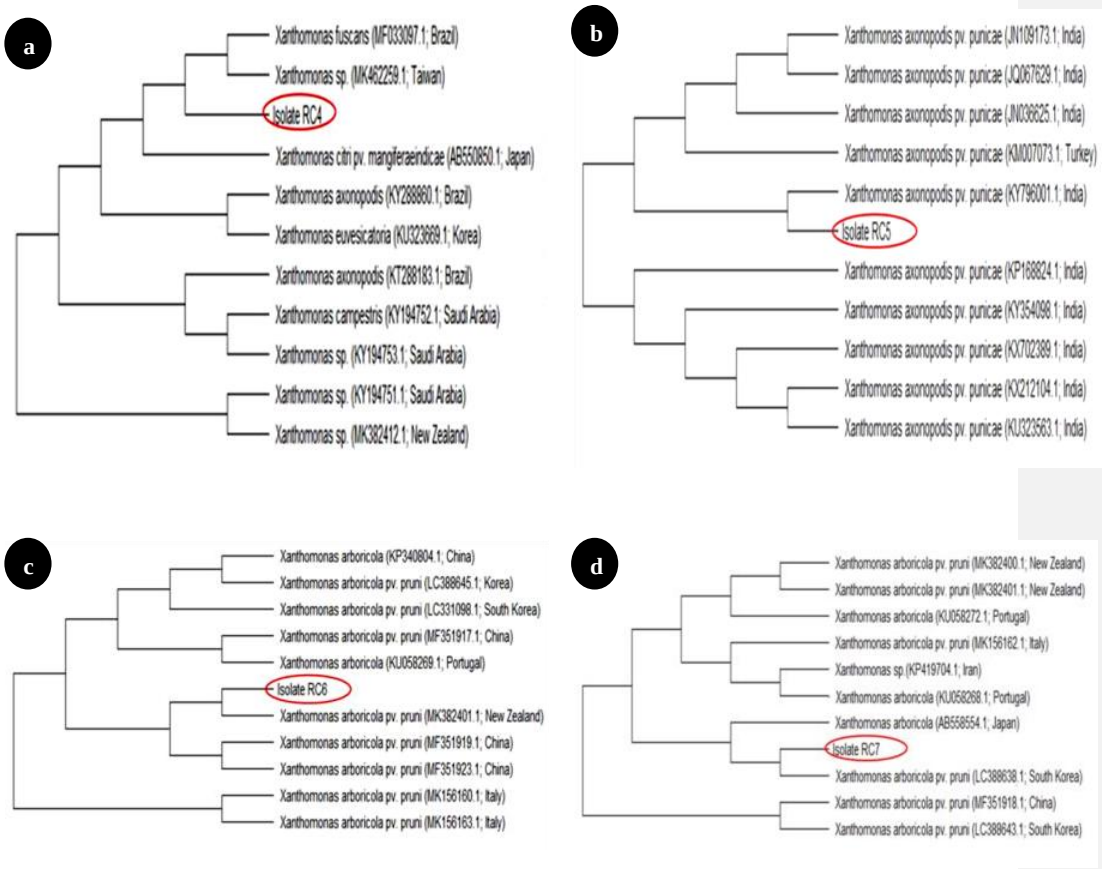

Fig. 6 Phylogenetic tree showing the genetic relationship of the bacterial isolates (*Xanthomonas* spp.)with other strains by using maximum likelihood method with 500 bootstrap replicates.
RC4 -Mango isolate *X. citri* pv. *mangiferaeindicae*, RC5 -Pomegranate isolate *X. axonopodis* pv. *punicae*,
RC6 -Peach isolate *X. arboricola* pv. *pruni*, RC7 -Plum isolate*X. arboricola* pv. *pruni*