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Isolation and Identification of Bacterial Pathogens in Tea (*Camellia sinensis*) and Coffee (*Coffea arabica*) Plants in Assam

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ABSTRACT

In India, plantation crops are considered an important part of the economy that plays an important role in agricultural and industrial development. Assam is the largest tea producer in India and coffee plantations are also there to some extent. Bacterial diseases are a major risk factor for crop losses that result in loss of productivity leading to a decline in the farm income of potential growers. Although bacterial diseases cause significant losses to plants in various locations of our country, however, the work done on these bacterial diseases in Assam as well as in other northeastern states is very scanty. Therefore, the present study was made to isolate, characterize and identify some of the bacterial pathogens associated with diseases of plantation crops in Assam. Suspected diseased samples from plantation crops viz., Tea (*Camellia sinensis*) and Coffee (*Coffea arabica*) were collected from Jorhat and Dibrugarh districts of Assam. On the basis of cultural, morphological, biochemical studies the two bacterial isolates were identified as the genus *Agrobacterium* (in Tea) and *Pseudomonas* (in Coffee). Further molecular characterization and the phylogenetic analysis was done to identify the different isolates i.e., isolate RC1 (Tea) as *Agrobacterium tumefaciens* (crown gall disease) and isolate RC2 (Coffee) as *Pseudomonas syringae* (bacterial blight of coffee).

Keywords Bacterial diseases, Plantation crops, Molecular characterization, *Agrobacterium*, *Pseudomonas.*

INTRODUCTION

Plantation crops are an important part of the Indian economy and play an important role in agricultural and industrial development. India is the leading country in the production of certain plantation crops in the world, e.g. Tea, Cashew, Arecanut, Coconut, Rubber and Cocoa. Of these major crops, India it is the seventh largest coffee producer in the world with a production of 316,000 MT in 2017-18 while the total area is 454.72 thousand hectares in 2017-18 (https://www.teacoffespiceofindia.com). Assam having a diverse climate, is favorable for growing many plantation crops. The crops found in this state are tea, coffee, rubber, arecanut, coconut and cashewnut. Among these important plants are tea, coffee, and rubber. Assam is the largest tea producer in India.

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In the mid-nineteenth century, tea was introduced as an industrial plant in Assam and Tripura. During the 1960's coffee was introduced in NE region. Steps are being taken by The Assam Plantation Crops Development Coorporation to establish coffee and rubber plantations in the North Cachar and Karbi Anglong districts of Assam (Baruah *et al*. 2008). However, the total area under planting plants in Assam during 2017-18 was 95.50 lakh ha and production was 190.64 lakh MT (Indian Tea Board 2018).

As such, the production of agricultural crops has helped to improve the state of the national economy but the incidence of some important diseases is considered to be major obstacles to achieving the potential yield of these important commercial crops. Although bacterial diseases cause significant losses to such plants in several regions of Assam, however, no systematic research has been conducted for identification, characterization and management of these diseases. The identification and characterization can help to understand a specific disease problem and will help to control the pathogen associated with the disease. However, detail information their complete characterization has not been done. The current study is made for isolation, identification and characterization of bacterial pathogens associated with diseases of plantation crops.

MATERIALS AND METHODS

Sample collection and isolation of the pathogens

Diseased samples from various infected parts like leaf, stem were collected from Jorhat and Dibrugarh districts of Assam. The collected samples were brought to the Department of Plant Pathology, AAU, Jorhat, where they had been further studied in the laboratory. The symptoms in tea (*Camellia sinensis*) was seen on the stem as rough roundish to irregular galls (Fig. 1). In coffee (*Coffea arabica*) symptoms were seen as necrotic lesion emerging from the margin with yellow halo in diseased leaves (Fig. 2). In case of isolation from galls, the method described by Islam *et al*. (2010) was followed. The sample was rinsed with tap water for removing the soil and other materials. Galls were sterilized with ten per cent household bleach (Salvon) for 1.5-3.0 min according to the nature of the galls. After washing three more times with sterile distilled water, galls were finely chopped and immersed in sterile water in a test tube and incubated overnight at room temperature (27-30°C). Overnight incubated crown gall extracts were streaked on YEMA media and incubated at 28±2°C for 48 h. The cultures so obtained were stored in the refrigerator at 4°C, which served as a stock culture for further studies. While in case of coffee, the pathogen was isolated following the standard protocol in Nutrient Agar (NA) and King's B medium. The pure colonies obtained were again streaked on NA, King's B and YEMA slants and kept for incubation at 28 ± 2°C for 24-48 h. The isolates were then preserved in the refrigerator at 4°C for future use.

Pathogenicity test

Pathogenicity tests were conducted using the pure culture of the isolates. The isolate from coffee was

Fig. 1. Roundish rough-surfaced galls on diseased stem of Tea (*Camellia sinensis*).

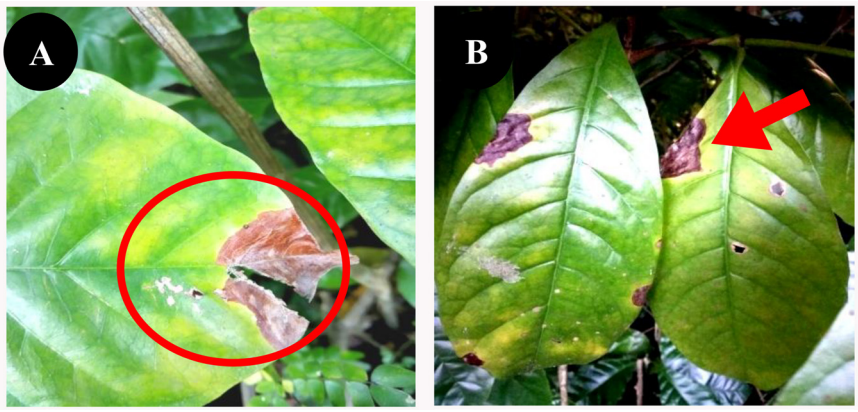

Fig. 2. (A) Necrotic lesion emerging from the margin with yellow halo in diseased coffee (*Coffea arabica*) leaves, (B) Necrotic lesions growing inward in V-shaped manner.

inoculated in the host plants by injection infiltration method (Klement 1963). Bacterial inoculum was taken in disposable injection syringe of 0.5 ml capacity. Care was taken that no air bubble was trapped in the suspension. The needle of syringe was placed on lower surface of the leaf, particularly at interveinal region and gently inoculum was injected. The leaf was firmly hold by providing support on the opposite side at the point of injection (mouth of the needle) with the help of index finger (left hand), while the thumb (left hand) was placed on the lower side above the mouth of the needle. The inoculum was infiltrated slowly by pressing the syringe with the thumb (right hand). Bigger wounds were avoided at the point of injection. Inoculated plants were kept in humid condition under shade house. After infiltration, plants were periodically monitored and observed for typical symptoms. Symptoms appeared within 7-10 days of inoculation. One seedling for the host plants were taken as control and inoculated with sterile water.

Stem inoculation technique was followed (New and Kerr 1972) in case of the isolate from tea. Seventy-two hours old growth of individual isolate on YEMA slants was vigorously shaken after adding 10 ml sterile distilled water in each slant and it gave approximately 108 cfu ml^{-1}. The host plants were wounded at stem to a depth of 3 mm with a blunt cylindrical sterile steel rod of 2 mm diameter. With the help of a sterilized micropipette 0.004 ml suspension of the isolate was deposited in the wounds. The wounds were immediately wrapped with sterilized non-absorbent cotton and were examined for the presence or absence of galls after 4 weeks.

Characterization of the bacterial isolates

Cultural, morphological and biochemical characterization of the isolates

Isolates showing positive results in the pathogenecity test were studied for the morphological characteristics using both simple and electron microscopy. 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 recorded. Biochemical characters such as oxidase, catalase activity and nitrate reduction, H^2S production and levan production were studied. In addition, carbohydrate utilization tests such as citrate utilization, starch hydrolysis, arginine utilization, indole, gelatin liquefaction, lysine utilization, ornithine utilization, urease, phenylanine deamination, lactose, glucose, galactose, L-arabinose, sorbitol, malonate, rhamnose, melibiose, sucrose, inositol, mannitol, erythritol, melezitose and esculin hydrolysis were conducted on the biochemical test kit (KB002 and KB009; Himedia, India).

Isolation of DNA, molecular and phylogenetic analysis

Genomic DNA of the bacterial isolates was done

by adopting the modified method of Cardinal *et al*. (1997). For 16S rDNA amplification, universal primers U16SF-5-AGAGTTTGATCMTGGCTCAG-3 and U16SR-5-TACGGYTACCTTGTTACGACTT-3 were used. PCR was performed in 10 µl PCR reaction [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); 3.5 µl Sterile distilled water] 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 resolved in 1.5% agarose gel. PCR sample were loaded similar to genomic DNA and run at 10-15 V/cm. After that result was observed in Gel Doc. The PCR product of the samples are sent to AgriGenome Labs Private Limited, Kerala and Bioserve Biotechnologies Private Limited, Hyderabad respectively for 16sRNA sequencing. The FASTA files are obtained from the 16sRNA results. Sequence similarity tool BLAST is employed to find the similarity of the sequences with known 16sRNA sequences in the GenBank database of NCBI to attain the accession numbers. Phylogenetic analysis of the isolates were carried out based on 16sRNA sequences of the isolates along with the sequences of other similar strains obtained from the NCBI GenBank. The sequences were aligned with Muscle align software using default parameters and a maximum likelihood tree was constructed using MEGA-X (Molecular Evolutionary Genetics Analysis) software with Kimura-2 parameter correction and 500-step bootstrap (Kumar *et al*. 2018).

RESULTS AND DISCUSSION

Morphological and cultural characteristics of the bacterial isolates

The morphological characters of the isolates were studied using Carl Zeiss Sigma Field Emission Scanning Electron Microscope. The shape of the isolates were ascertained to be rod shaped. The colony characters of the bacterial isolates were studied on different media such as nutrient agar, King's B medium and YEMA medium. The isolate from tea produced white, circular, convex, entire colonies and were 5 mm in diameter (Table 1). Whereas, the isolate from coffee produced creamish, opaque, circular to irregular, convex, mucoid colonies with size ranging from 2.5-3.5 mm in diameter (Table 1).

Pathogenicity tests

For proving the pathogenicity and to satisfy the Koch's postulate, stem inoculation was done (New and Kerr 1972) in case of tea (*Camillia sinensis*) and for coffee (*Coffea arabica*) the pathogens were inoculated in the host plants by injection- infiltration method (Klement 1963). The appearance of gall was observed four weeks after inoculation of the isolate from tea in the plant near the soil region. Initially

Table 1. Morphological, cultural and pathological characteristics of the bacterial isolates. (+) = positive; (-)= negative.

	Sample code	RC1 (Tea)	RC2 (Coffee)
Morphological characters	Size (µm)	0.7x2.3	0.5x2.3
	Shape	Rod	Rod
Colony characters on plate	Colony shape	Circular	Circular to irregular
	Surface	Smooth, Mucoid	Smooth, mucoid, glistening
	Edge	Entire	Entire
	Color	White	Cream
	Elevation	Convex	Convex
	Opacity	Translucent	Opaque
	Size (mm)	5	2-3
Cultural characteristics	Gram reaction	(-)	(-)
	Pigment production In King's B medium	(-)	(+)
	KOH test	(+)	(+)
	Oxygen requirement	Aerobic	Aerobic
Pathological test	On host crop	(+)	(+)

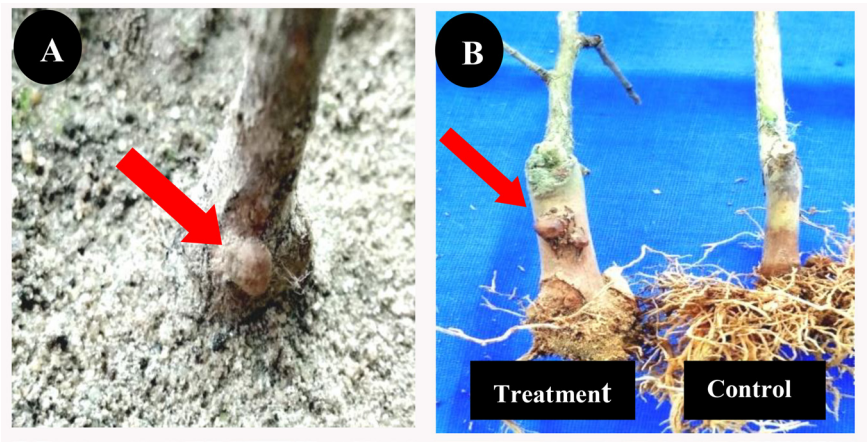

Fig. 3. (A) White roundish galls produced by the isolate RC1, 4 weeks after inoculation. (B) Rough dark brown galls observed on the inoculated plant.

the galls were creamy colored but later they turned rough, dark brown. After reisolation the pathogen was found identical to the original culture (Fig. 3). Similar symptoms were observed in case of other crops by

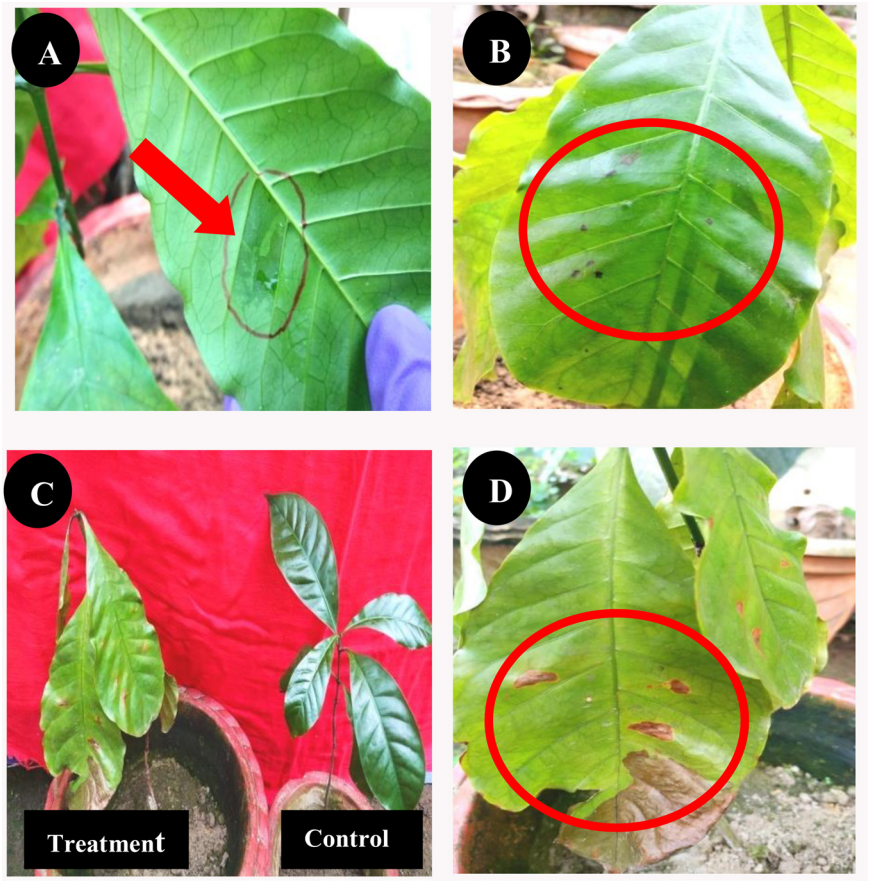

Fig. 4. (A) Inoculation of bacterial suspension of isolate RC2 by injection infiltration method; (B) Appearance of water soaked spots within 5 days of inoculation; (C) Appearance of typical symptoms on the treated plant 12 days after inoculation; (D) Plant showing necrotic area with yellow halo.

Furuya *et al*. (2004), Mattysse (2006), Burr and Katz (1983), Gupta *et al*. (2013). In the coffee plants water-soaked spots appeared within 5 days of inoculation of the pathogen. After 12 days of inoculation with the pathogen, the coffee seedlings showed exactly similar with the original blight disease symptoms, caused necrotic lesions at the inoculation site and showed a light-brownish, enlarging area surrounded by a yellow halo (Fig. 4). Similar symptoms were described by Korobko and Wondimagegne (1997), Ito *et al*. (2008), Mugiira *et al*. (2011), Hinkosa *et al*. (2017). Reisolations from symptomatic plants yielded bacterial colonies that were identical to those used for the inoculations and the control plants did not showed any symptoms.

Biochemical characterization of the bacterial isolates

The results obtained on various biochemical characteristics of the bacterial isolates are presented in (Table 2). The results indicated that both the isolates from tea and coffee were KOH positive. Biochemical test results of catalase and levan production were found to be positive for both the isolates and showed negative for nitrate reduction and H_2S production. Whereas, isolate from tea showed negative result for oxidase and coffee isolate showed positive result. In addition, carbohydrate utilization tests such as citrate utilization, indole, arginine utilization, gelatin liquefaction, L-arabinose, galactose and malonate melibiose were found to be positive for both the isolates (tea and coffee). Whereas for sucrose, inositol, sorbitol, melezitose, phenylalanine deamination and lysine utilization tests were found to be negative in both the cases. The coffee isolate showed positive result for starch hydrolysis, erythritol, glucose and mannitol utilization. But for the tea isolate, these tests were negative. Also the isolate from tea showed positive result for lactose, urease, rhamnose, ornithine utilization and esculin hydrolysis. Whereas, the isolate from coffee showed negative result for these tests. All the biochemical characters and carbohydrate utilization under present study were co-related with the characters for all the isolated pathogens reported by several workers. Setti and Bencheikh (2013), Gupta *et al*. (2013), Rouhrazi and Rahimian (2014), Raghu Ram (2016), Spiers (2011), Islam *et al*. (2010) and Yaram *et al*. (2015) observed similar results in case of the bacterial pathogen isolated from several crop plants, where they reported the pathogen to be *Agrobacterium tumefaciens*. Likewise, Hinkosa *et al*. (2017) and Belan *et al*. (2016) found similar results on the isolate from coffee that showed similar characteristics with *Pseudomonas syringae* causing bactetial blight in coffee. Similar findings were described in the Bergey's Manual of Systematic Bacteriology (Buchanan and Gibbons 1984) for the isolated bacterial pathogens respectively.

Table 2. Results for biochemical characters and carbohydrate utilization of the isolates. (+) = positive; (-)= negative.

Biochemical tests	Samples	
	RC1 (Tea)	RC2 (Coffee)
Gram staining	-	+
Catalase	+	+
Oxidase	+	-
Production of H_2S	-	-
Nitrate production	-	-
Levan production	+	+
Carbohydrate utilization		
Sucrose	-	-
Mannitol	-	+
Sorbitol	-	-
Indole	+	+
Inositol	-	-
Melibioze	+	+
Galactose	+	+
L-Arabinose	+	+
Rhamnose	+	-
Lactose	+	-
Glucose	-	+
Urease	+	-
Malonate	+	+
Erythritol	-	+
Melizitose	-	-
Esculin hydrolysis	+	-
Arginine utilization	+	+
Lysine utilization	-	-
Citrate utilization	+	+
Ornithine utilization	+	-
Gelatin liquefaction	+	+
Starch hydrolysis	-	+
Phenylalanine deamination	-	-

Molecular characterization and phylogenetic analysis

The bacterial isolates obtained from diseased plants were subjected to molecular identification. PCR analysis of the extracted DNA was done using uni-

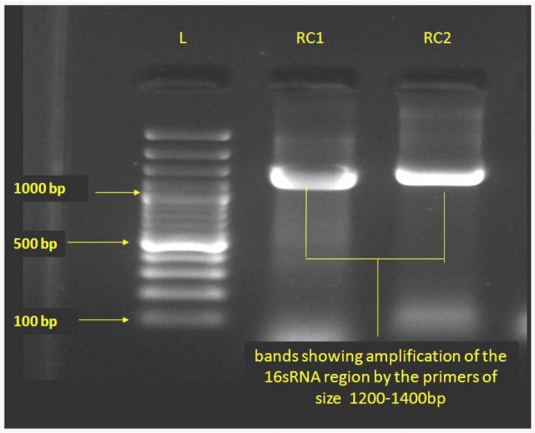

Fig. 5. Agarose gel Electrophoresis Showing Amplification of the Bacterial DNA Products. L=100bp Ladder. RC1 and RC2 = Bacterial Isolates.

versal primers (forward US16F and reverse US16R). The amplicons were subjected to agarose gel (1.5%) electrophoresis which showed a clean amplification of~1200-1400 bp fragments of 16S rDNA genes (Fig. 5). The BLAST results showed maximum homology of the bacterial isolates with 10 different strains of the respective genera. Sequence comparison of the 16S rDNA gene of the isolate from tea with GenBank entries further confirmed the identity as the similarity percentage was 100% (AB68027.1, Japan) to that of *Agrobacterium tumefaciens* entries in the database. The isolate from coffee showed highest homology with the strains of *Pseudomonas syringae* with 97% similarity (AF130950.1, USA).

For construction of the phylogenetic tree MEGA-X software (Kumar *et al*. 2018) was used. The phylogenetic analysis was carried out using partial 16S rRNA sequences which revealed the phylogenetic position of the isolates. The results showed that the isolate from tea showed homology with the strains of *Agrobacterium tumefaciens* (Fig. 6) and isolate from coffee showed homology with the strains of *Pseudomonas syringae* (Fig. 7).

There is a tremendous scope for large scale expansion of different plantation crops in Assam which will have considerable impact on the state's economy. Different pest and diseases cause significant production loss of these crops leading to decline in the farm income of the potential growers. Therefore, it is of utmost importance to tackle down the problems faced due various diseases. Plant diseases caused by bacteria are commercially important worldwide for agriculture. Besides bacterial pathogens that are already established in many areas, there are also many instances of emergence and re-emergence of new pathogen variants. The present research has put forwarded the identification and characterization of the bacterial pathogens associated plantation crops of Assam. It has thrown some light regarding the occurrence of bacterial diseases and its importance in plantation crops. Since Assam as well as other North Eastern states have a favorable climate and holds

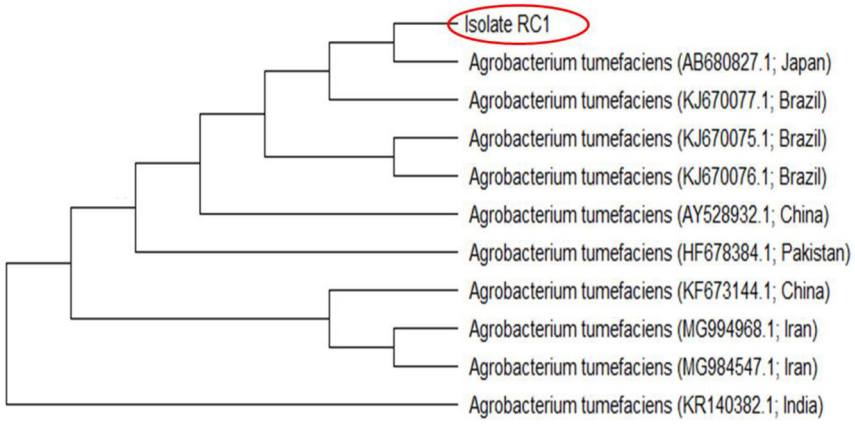

Fig. 6. Phylogenetic tree showing the genetic relationship of the isolate RC1 to other strains by using maximum likelihood method with 500 bootstrap replicates.

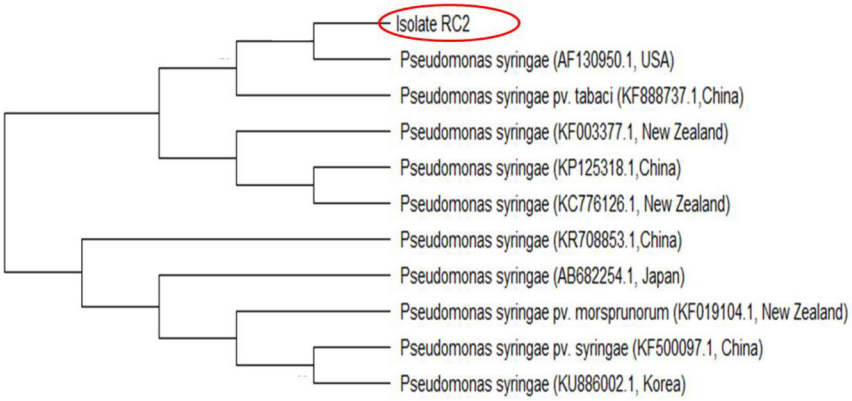

Fig. 7. Phylogenetic tree showing the genetic relationship of the isolate RC2 to other strains by using maximum likelihood method with 500 bootstrap replicates.

a high potential for cultivation of different crops, therefore more elaborate studies regarding bacterial taxonomy is necessary. So, detailed study covering almost all the diseases of plantation crops of Assam including North East region required to know the biodiversity of these plant pathogenic microflora so that proper management strategy can be applied.

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