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Phylogenetic Analysis of Bacteria involved in Different Diseases of Tomato (*Solanum lycopersicum* L.) Crop in District Charsadda, Khyber Pakhtunkhwa-Pakistan.

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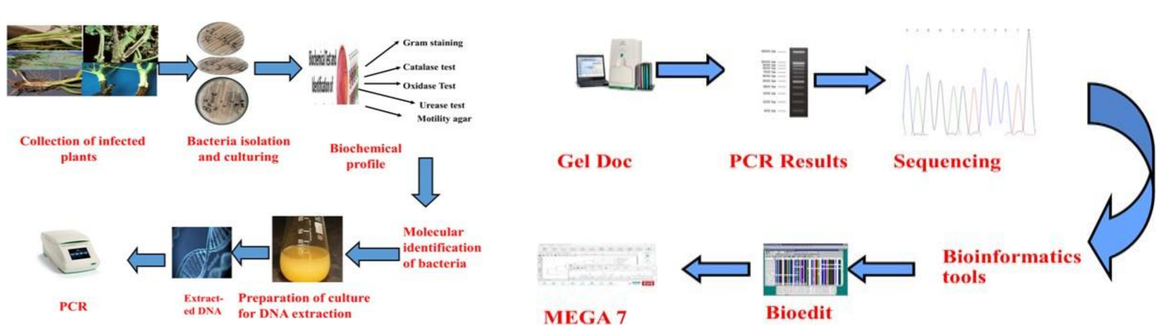
Abstract

Tomatoes are exposed to potential microbial contamination. The objective of the current research was to isolate and identify the causal agent of bacterial canker, bacterial wilt, pith necrosis, leaf tip chlorosis, stem rot, and bacterial leaf spot disease on tomatoes in district fields of Charsadda. We collected 50 random samples from different fields in district Charsadda and were cultured on the nutrient agar media and nutrient broth for bacterial growth. Further culture on selective media and biochemical techniques together with molecular sequence analysis was used to probe the particular microbe. A total of 40 samples showed growth on nutrient agar and nutrient broth media. After the biochemical test, the data was analyzed through ABIS software, and isolates were identified as *Ralstonia solanacearum*, *Clavibacter michiganensis*, *Xanthomonas vesicatoria*, *Pectobacterium carotovorum, and Pseudomonas corrugata.* The isolates were further analyzed using *16S rRNA* gene-based sequence analysis. We found that isolates consist of five main species *Ralstonia solanacearum*, *Clavibacter michiganensis*, *Xanthomonas vesicatoria*, *Pectobacterium carotovorum*, and *Pseudomonas corrugata.* It was concluded that different bacteria were involved in different tomato diseases like *Clavibacter michiganensis* in bacterial canker, *Ralstonia solanacearum* in bacterial wilt, *Xanthomonas vesicatoria* in bacterial leaf spot, *Pseudomonas corrugata* in pith necrosis and leaf tip chlorosis, and *Pectobacterium carotovorum* in stem rot diseases.

KEYWORDS

Bacteria, Pathogens, Tomato, *16SrRNA*, Necrosis

Graphical Abstract



1.0 INTRODUCTION

Solanum lycopersicum L. (Tomato) originated in the Andean region of South America and belongs to the family *Solanaceae*. It is a widely cultivated vegetable crop throughout the world and ranks next to the potato in world acreage (*Agarwal* et al., 2020). It is the most processing crop and ranks first among the vegetables. Since the mid-nineteenth century, tomato farming has become much more widely known due to its diverse climate adjustability as well as good nutritional value. China is one of the most tomato's producing countries followed by the United States and others (CIA 2017). There are several factors involved in the successful cultivation of quality tomatoes of which diseases caused by abiotic factors, bacteria, fungi, nematodes, and viruses play an important role (Blanchard, 1992). Fungal diseases that affect the yield of tomatoes are septoria leaf spot (*Septoria lycopersici Speg.)*, wilt (*Fusarium oxysporum f. sp. lycopersici (Sacc.)*, early blight (*Alternaria solani*), buckeye rot (*Phytophthora infestans (Mont.)*), and fruit rots (Sokhi et al., 1991). Diseases such as bacterial spots and bacterial canker in tomatoes in rainy conditions or under moist conditions are caused by bacterial species *Clavibacter michiganensis subsp. michiganensis* and *Xanthomonas campestris pv. vesicatoria* (Jones et al., 1991; Chang et al., 1992; Bouzar et al., 1994 and Quezado-Duval et al., 2004). There is a significant reduction in the yield of tomato and pepper due to bacterial speck (*Pseudomonas syringae pv. tomato*) and bacterial spot (*Xanthomonas spp.)* diseases. Darklesions on the fruit and foliage of plants were produced by *Pseudomonas syringae pv. Tomato* and *Xanthomonas spp.* (Potnis et al., 2015).

The present study is designed to identify different bacteria involved in tomato plant disease through molecular and biochemical screening. Also, to conduct phylogenetic analysis of identified bacteria through 16s RNA sequencing.

2.0 MATERIALS AND METHODS

2.1 Plant Collection and Extraction

Collection of samples based on symptoms

Leaf, stem, root and fruits were collected based on the physical appearance of symptoms like the spot on leaves, leaves with yellow and brown margins, limb dieback with rough canker on stem and amber-colored on fruit, etc. A total of 50 samples10 for each part were collected from District Charsadda and were transferred to Biotechnology Lab in Bacha Khan University Charsadda.

Processing of samples

The Collected samples were washed with distilled water and the purified samples were then inoculated on nutrient Agar media using the streaking plate method. The samples were also cultured in nutrient broth media. After inoculation, the plates were placed at 37 OC for 24 hrs. Upon the success full growth, samples were sub-cultured under the same conditions for another 24 hours at 37 ^{O}C.

Identification of Isolates:

These isolates were identified based on morphology, biochemical and molecular techniques. Morphological identification was done from bacterial colony colors, shape on culture media and to differentiate bacteria between the gram-positive and gram-negative. Gram staining of the bacterial cell wall was followed. Biochemical identification was performed through different tests, i.e. Catalase, Oxidase, Urease, Motility Tests.

Biochemical Analysis through ABIS:

Biochemical data like citrate utilization, Indole production, urease, oxidase, and catalase production were analyzed through ABIS (Advance bacterial identification software) which identified bacteria based on these characteristics.

Preparation of culture for DNA extraction:

Bacteria were cultured on nutrient broth media for isolation of DNA. The culture was stored at a low temperature for genome isolation. The standard protocol of (Paneto et al., 2007) was used for DNA isolation. After DNA extraction, DNA was run on agarose gel electrophoresis for visualization of bands and was quantified by a Nano spectrophotometer. All the samples were brought to the same concentration by the addition of elution buffer. Agarose gel pictures were saved as a reference for further process.

PCR Amplification:

16S rRNA primers were used for species-level bacterial identification. Reported primers were used for PCR amplification e.g. Forward primer<AGAGTTTGATCMTGGCTCAG>While thereverse primer was 1492R <TACGGYTACCTTGTTACGACTT>. PCR conditions were optimized for different concentrations of template DNA primers, $MgCl_2$, Taq Polymerase enzyme, the volume of DNA, and the annealing temperature (50-60˚C) of primers. DNA with the help of Touchdown PCR was amplified to obtain a large number of DNA clone segments. The process was performed out through 35 cycles in 2 successive stages of 10 and 25 cycles, as shown below. PCR products had been analyzed for confirmation of amplification using1.2 % agarose gel electrophoresis.

The collected PCR products were washed with ethanol and sequencing was done using Sanger sequencing to ABI genetic analyzer 3130. The obtained sequencing data were analyzed by Finch TV 1.4 and codon code aligner. To recognize variations at a time in all sequences obtained, multiple sequence alignment was done by CLUSTAL W and the phylogenetic tree was constructed by the MEGA 7 program basis.

3.0 RESULTS AND DISCUSSION

In this research, 50 total infected plant parts like stem, root, pith, leaves, and fruits were randomly collected from district Charsadda. Various cultural media were utilized for bacterial growth, such as Nutrient Agar, and Nutrient broth media. The media was prepared via autoclaving for 15 min at 121˚C, to make it germ-free and was then transferred to the sterilized dishes after sterilization in a sterile condition. Obtained plant samples were then inoculated on the media plate having nutrient agar to create a growth environment for bacteria after media preparation, and the plates were incubated at 37 ˚C for 24 hours. The growth of bacteria on40 plates was noted after 24 hours, as given in Fig.1.

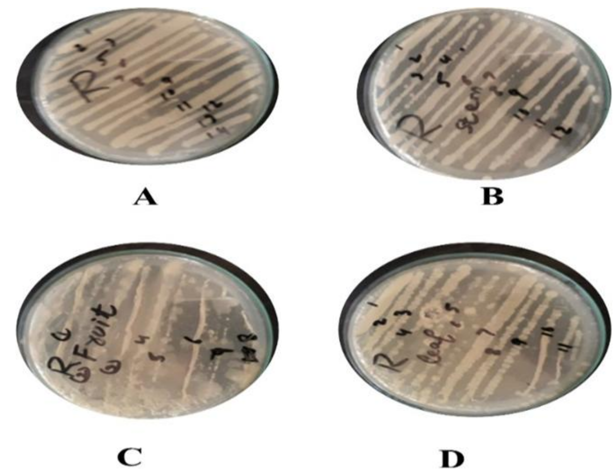


Fig.1. Petri plates containing samples from infected roots, stems, fruit, and leaf. **A.** Refers to the positive growth of root isolates. **B**. Refers to the positive growth of stem isolates. **C.** Refers to the positive growth of fruit isolates. **D.** Refers to the positive growth of leaf isolates.

Biochemical Analysis

After the observation under microscopic, 6 samples were gram-positive and 34 were gram- negative. The catalase test showed 32 species were catalase positive while the 8 were catalase- negative. The Oxidase test showed that the 32 samples were oxidase-positive while the 8samples were oxidase negative. The urease test showed that the 26 samples were urease positive and the 14 samples were urease negative. The motility test showed that the 06samples were non- motile while the 34 samples were motile and have flagella. Details showed in Fig.2. and Table 1.

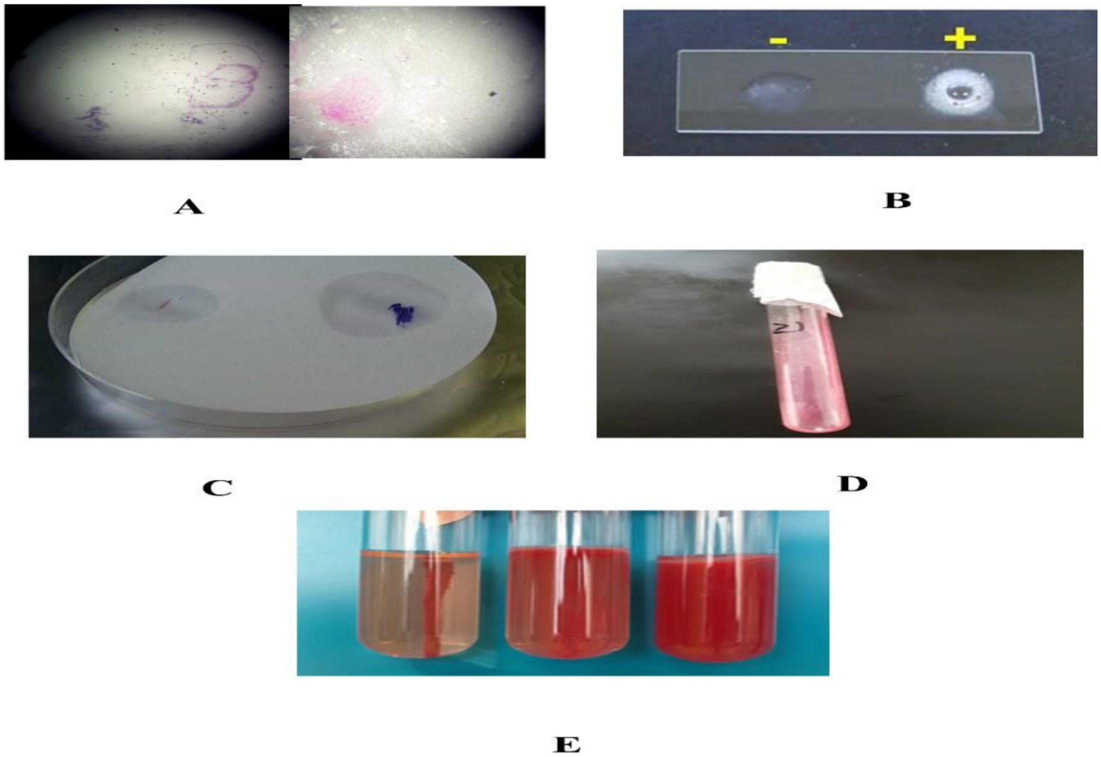


Fig.2. The results of Gram Staining, catalase, oxidase and urease, motility test on plants isolates: **A**. Related to the negative result and positive result of Gram Staining test. **B**. Refers to the positive and negative result of catalase test. **C**. Refers to the positive and negative result of oxidase test on plants isolates. **D**. Refers to the positive result of Urease. **E**. Related to the negative result and positive result of motility agar analysis on plants isolates.

Table1. Biochemical Characterizations of all samples

Biochemical Tests					
	Gram staining	**Urease**	**Oxidase**	**Motility**	**Catalase**
No of +ve samples	6	24	32	34	32
No of –ve samples	34	14	8	6	8

Biochemical Analysis through ABIS:

The biochemical test data were put into an online software ABIS and based on different characterizations identified different bacteria. Different isolates that were identified through ABIS software are *Ralstonia solanacearum*, *Clavibacter michiganensis*, *Xanthomonas vesicatoria*, *Pectobacterium carotovorum, and Pseudomonas corrugata* shown in Table 2.06 samples out of the 10 positive samples were *Clavibacter michiganensis* which were involved in bacterial canker disease. The 4 samples out of the 10 samples were *Ralstonia solanacearum* positive which was involved in bacterial wilt disease and the 8 samples out of the total 10 samples were *Xanthomonas Vesicatoria* positive which was involved in bacterial leaf spot disease. The 7 samples out of the 10 samples were *Pseudomonas corrugata* positive which were involved in pith necrosis and leaf tip chlorosis and the 8 samples out of the total10 samples were *Pectobacterium carotovorum* which were involved in the stem rot disease as shown in Table 2.

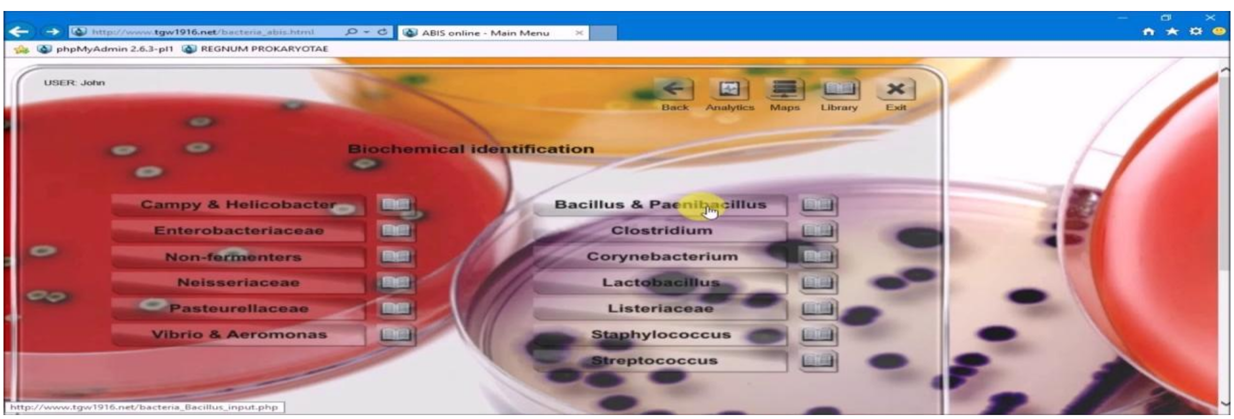


Fig.3. Identification of bacteria through ABIS online software

Table2. Distribution of Bacterial Pathogens Identified through ABIS

S. No	**Part of the infected plant**	**Disease**	**No. of Samples**	**Name of the bacteria**	**Count**
1	Stems & fruits	Bacterial canker	10	*Clavibacter michiganensis*	6
2	Roots	Bacterial wilt	10	*Ralstonia solanacearum*	4
3	Leaf	Bacterial leaf spot	10	*Xanthomonas Vesicatoria*	8
4	Leaf tip & Pith parts	Pith necrosis and leaf tip chlorosis	10	*Pseudomonas corrugata*	7
5	Stem	Stem rot	10	*Pectobacterium carotovorum*	8

Molecular identification of Bacteria:

The biochemical analysis identified five different species of bacteria involved in causing disease in different parts of the plant. These include *Ralstonia solanacearum*, *Clavibacter michiganensis*, *Xanthomonas vesicatoria*, *Pectobacterium carotovorum,* and *Pseudomonas corrugata*. Random samples from each group were selected for further molecular identification. DNA was extracted from each sample and was run on 0.8% agarose gel for visualization and confirmation.

Confirmation of DNA

3µl of bromophenol blue was mixed with 2µl of isolated DNA and was applied to the wells shaped within gel; bromophenol blue serves as a tracking and loading dye. The products were moved toa1 XTA Ebufferata fixed voltage of 80V for 30minutes of gel. The bands of DNA were noted using UV light and the band was recorded by a method of gel documentation. Gel images, used as a source for further procedures, have been saved.

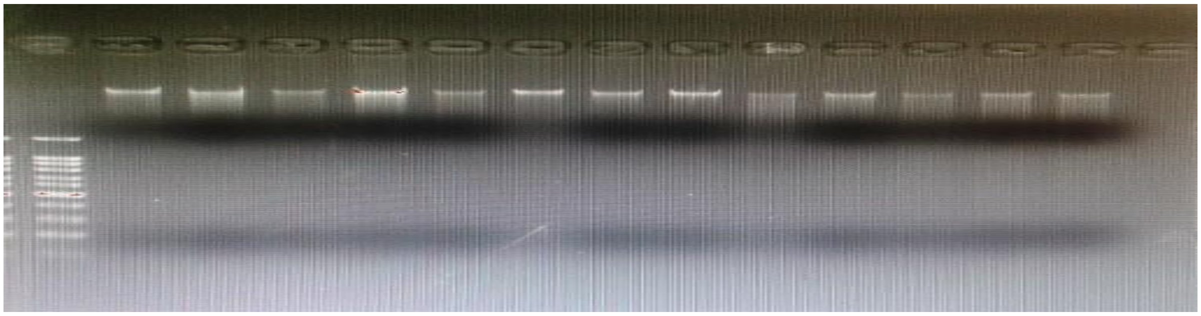


Fig.4. The gel image indicated DNA extracted from the Tomato plant samples.

QuantificationofDNAbyNanoDrop™2000/2000c Spectrophotometers

DNA was quantified by Nano Drop in which 22.67nanogram/µl was noticed as given in Fig.5.

Fig.5. Quantification of mDNA using Nano drop.

Polymerase Chain Reaction

Using Touchdown PCR, the bacteria's DNA was amplified to obtain more numbers of clones of a specific piece of DNA. Fig.6. demonstrates the amplification of the ideal 16S rRNA gene by Touchdown PCR.

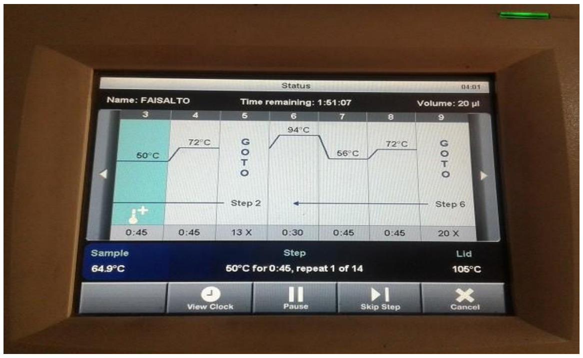


Fig.6. Gene amplification through Touch Down PCR

The PCR products were run for conformation on 1.2 to 2.0 % agarose gel to confirm DNA. As a reference on the first well of gel, a 100bp DNA ladder was also run. The PCR products obtained were examined utilizing UV light as a fluorescent band of the expected size and recorded using the gel documentation method as shown in Fig.7.

Fig.7. Amplification bands of the16SrRNAgene

Sequencing of sample data and Phylogenetic Study

Using ABI genetic analyzer 3130the PCR products were sequenced. The obtained sequences were further analyzed using different bioinformatics, tools and the phylogenetic tree was constructed using MEGA-7software. Sample-1 is more closely related to Ralstonia *Solanacearum* strain k 60-1 and *Ralstonia Solanacearum* strain LMG 2299. While less related to the *Ralstonia solanacearum* strain LMG 2302 and *Ralstonia solanacearum* strain INTABV18 as showninFig.8. Sample-2 was more related to the *Xanthomonas campestris* pv Campestris strain Xcc-C7 and was less related to the *Xanthomonas cynarae* strain CFBP 4188 shown in Fig.9. Sample 3was more closely related to subsp of NR 037135.1 *pseudomonas corrugata* strain Slade 939/1.While it was less related to LC420200.1Pseudomonassp. Ls9 gene shown in Fig.

10. Sample 4 was more related to the subsp of NR 041971.1 *Pectobacterium carotovorum* strain DSM 30168 and was less related to the MG890366.1 *Pectobacterium carotovorum* strain 8694 shown in Fig.11. Sample 5 was closely related to the subsp of KX373436.1 Clavibacter michiganensis strain PF007 and was less related to KX373415.1 *Clavibacter michiganensis* strain 4641 shown in Fig.12.

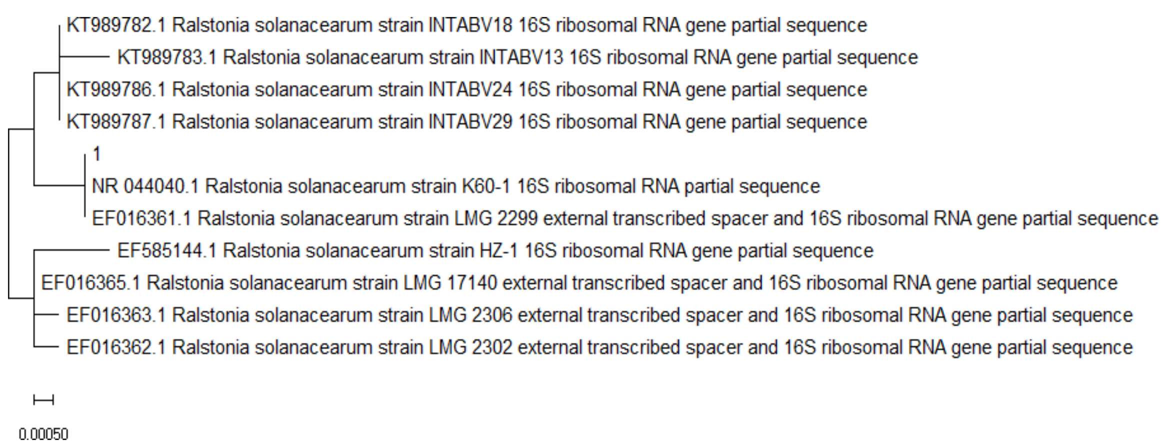


Fig.8. The phylogenetic tree of Sample No. 1

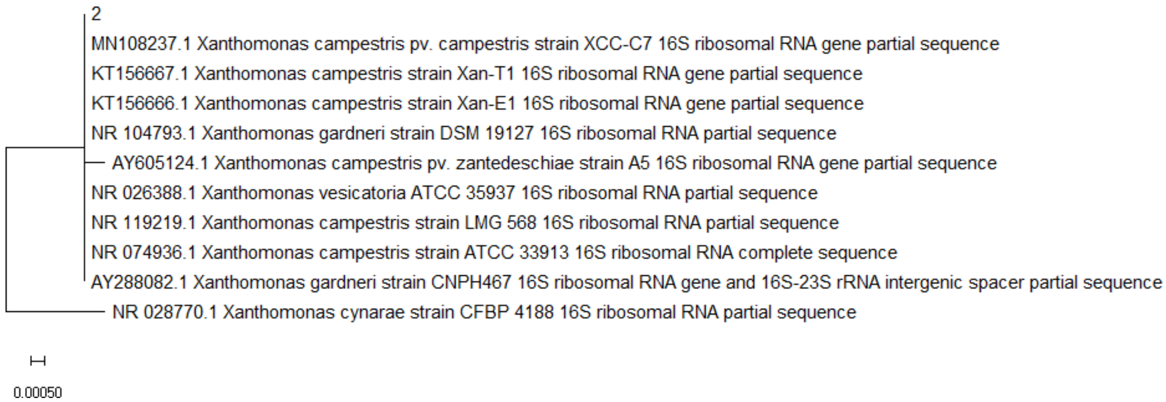


Fig.9. PhylogeneticanalysisofSampleNo. 2

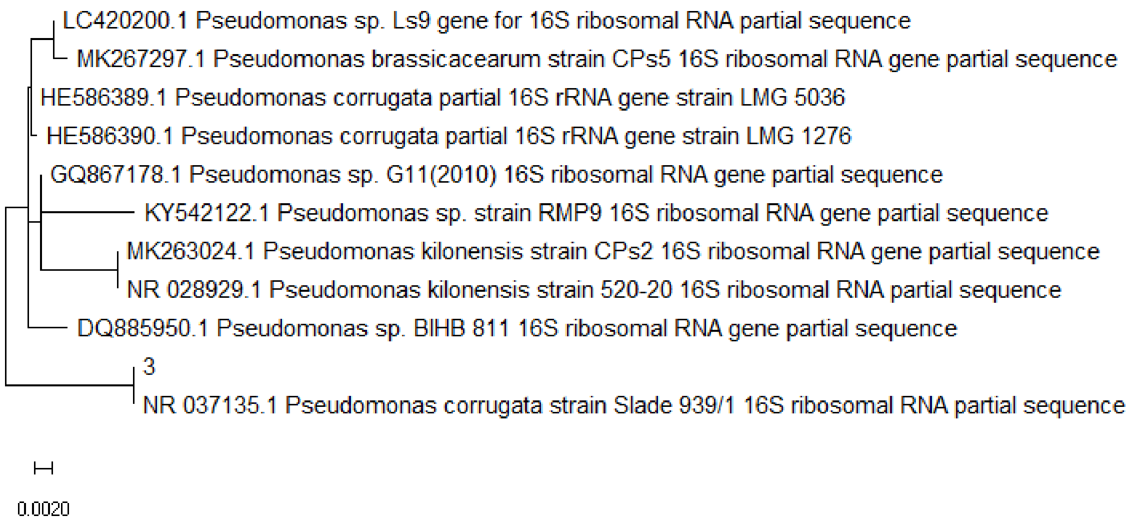


Fig.10. Phylogenetic analysis of Sample No. 3

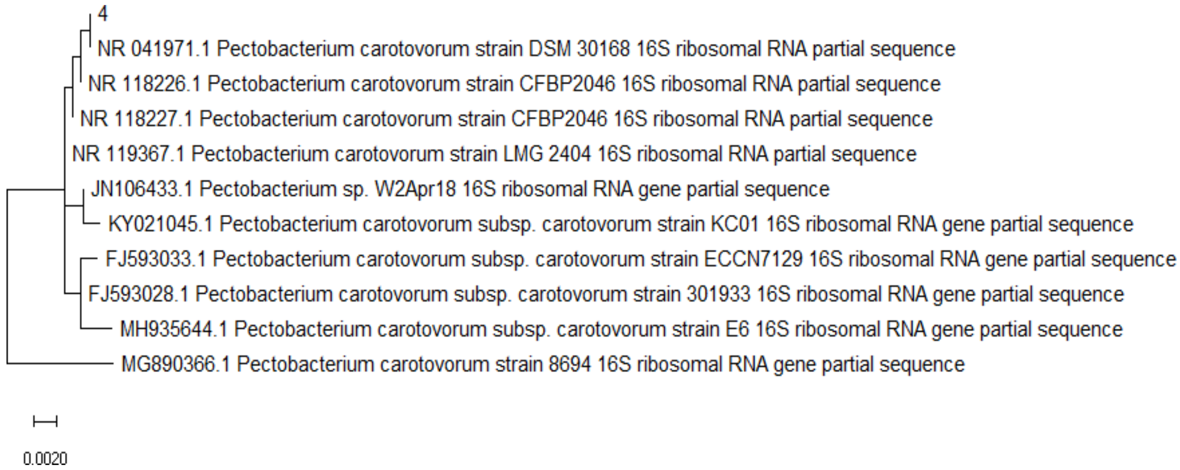


Fig.11. Phylogenetic analysis of Sample No. 4

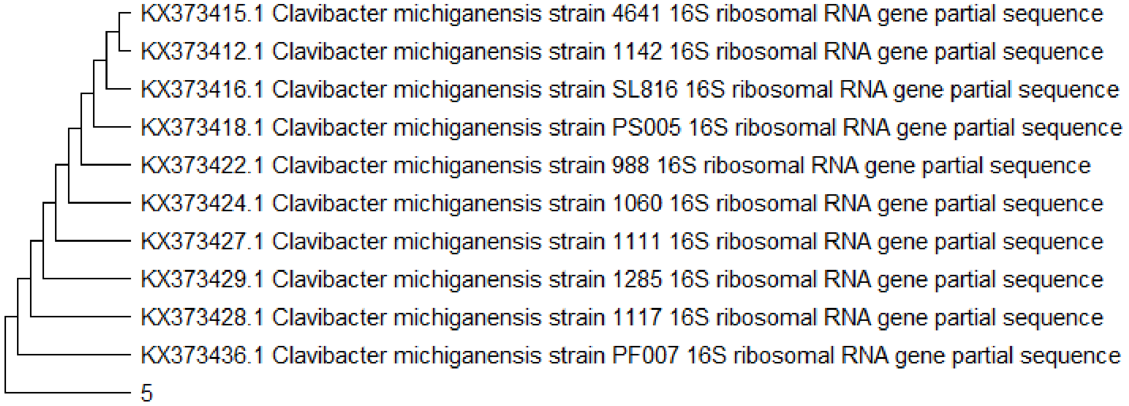


Fig.12. Phylogenetic analysis of Sample No. 5

4.0 CONCLUSION

In conclusion, *Conyza canadensis* showed a high urease inhibitory activity, particularly in butanol and methanol fractions. Such fractions can be potential sources of natural urease inhibitors and can potentially be used in the treatment of Helicobacter pylori infection and other gastrointestinal diseases. Further research must be done to isolate the active compounds and identify their therapeutic potential and safety to be applied to clinical practice

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CONFLICT OF INTEREST

The authors declare no conflict of interest

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