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AN UNCULTURED BACTERIUM ASSOCIATED WITH INFECTION IN MANDARIN (*Citrus reticulata*) IN PAKISTAN

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ABSTRACT

This study was aimed to isolate and characterize the bacterium associated with citrus greening disease. As this disease cause losses in citrus industry of Pakistan. It was suspected that may be there are more than one bacterium involved in this disease development. This disease look like happens due to complex population of microorganism so the reason is still not identified. There is a need of extensive research on citrus greening to know the basic reason for this disease. Therefore an extensive survey of citrus orchards infected with citrus greening disease in Mian Chanu region of Punjab, Pakistan was conducted in August, 2015. Vein yellowing, blotchy mottle and vein thickening were recorded. . The PCR product of size 1500 bp was amplified with universal 16sRNA primers 27F/1492R and Nucleotide sequence was deposited in Gene Bank NCBI through accession number LT592134. BLAST and Phylogenetic analysis established their association with separate group. In our knowledge, this might be a new uncultured bacterium (1369 bp), associated with infection in citrus tree in Mian chanu, Pakistan.

Key word: Mandarin orange, uncultured prokaryote, clone MC1, 16sRNA.

INTRODUCTION

A small sized specialized bacteria also known as Phytoplasma. They lived mostly in food vessels of plants and spread from one plant to other plant by insect vector (Weintraub *et al.*, 2007). In 1967, phytoplasma were first discovered and named as Mycoplasma-Like Organisms (MLO) (Doi *et al.*, 1967). These organism cannot be cultivated in media (Jones, 2001; Harrison *et al.*, 2015), that's why identification of syndromes caused by phytoplasma is very problematic. These are liable for the beginning of a large number of infections in several plant species throughout worldwide (Liefting *et al.*, 2008). Lot of phytoplasmal infections have been reported in numerous vegetable plants (Tapia-Tussell *et al.*, 2012). Plants infected by phytoplasmas displays several noticeable symptoms of infection for example yellowing, leaf curling, little leaf, phyllody, witches' broom, severe arresting of plant growth, etc. (Bertaccini *et al.*, 2005) Various phyto plasma has been identified from different plants owing to the improvement of molecular techniques (Bertaccini and Duduk, 2010).

Citrus is the leading fruit of Pakistan and the greatest producer of Kinnow in the world (Siddique and Garnevska, 2018). Sargodha, Jhang and Toba Tek Singh are major citrus growing areas in central Punjab. Pakistan produced 2.4 million tonnes in 2015–2016. Its exports contribute significantly in the economy of country exporting about 15,912.8 thousand tons of citrus (GOP, 2017). Pakistan produce 2.5 million ton of kinnow. Punjab province produced (95%), Sargodha district shares major portion (80%) (Ashraf *et al.*, 2015). Citrus diseases are major cause of reduced yield and require immediate attention. Citrus diseases cause enormous losses every year to citrus industry of Pakistan. Citrus greening (HLB) has been established itself in more than 40 countries (Iftikhar *et al.*, 2016). HLB is caused by the fastidious Gram-negative uncultivable bacterium belonging to the a-subdivision of the phylum Proteobacteria (Garnier and Bové, 1996). Many scientist have been elaborated the symptomology of HLB (Bové, 2006; Batool *et al.*, 2007; Munyaneza, 2010). Typical symptoms include small and upright leaves and chlorotic mottling, Zn deficiency symptoms, severe vein yellowing and greening of mature fruits. The pathogen has not restricted itself to Citrus species. Several other hosts have also been identified (Jagtap *et al.*, 2013).

In India there is one report of chili leaf disease that is associated with a phytoplasma by (Singh and Singh, 2000; Win and Jung, 2012), and identified an aster yellows phytoplasma (Khan and Raj, 2006) infecting chili plant. According to our understanding, little work has been done for characterization and ultimate identification of phytoplasmas responsible for infection in citrus tree in Mian Chanu region of Punjab Pakistan. Therefore in current study, we make an attempt to identify and characterize the phytoplasma that is may be responsible for infection in citrus plants from Mian Chanu region of Punjab Pakistan.

MATERIALS AND METHODS

During the year 2015, infected citrus plants were exhibiting blotchy mottle, yellow veins vein thickening in Mian Chanu as compared to healthy citrus plant (Figure 1).

Figure 1: Healthy and infected citrus plants.

The DNA of leaf sample of citrus variety (UAF-7)was extracted with Cetyl trimethyl ammonium bromide (CTAB) method by Doyle, (1990). For amplification of DNA by PCR a reaction mixture of 25 µL containing 3 µL template DNA, 0.12µL 10X Taq polymerase buffer, 1µL mM dNTPs, 4µL mM MgCl2, 1µl of 10 pmol forward primer 27-F (5' AGA GTT TGA TCM TGG CTC AG 3') (Frank *et al.*, 2008), 1µl of 10 pmol reverse primer 1492-R (5' ACC TTG TTA CGA CTT 3'), d.H_2O 9.88 µl and 0.12 µl Taq DNA polymerase (Thermo Fisher Scientific) was prepared in a 0.25 mL thin walled PCR tube. The machine was programmed for a preheat treatment followed by 35 cycles of 94 ˚C for 5 mins, 94 ˚C for 1 min, 55 ˚C for 1 min and 72 ˚C for 1 min, followed by a final incubation of 10 mins at 72 ˚C. Standard protocol of Gene cloning kit (Thermo Fisher Scientific) was followed to clone the 1500 bp fragment in PTZ57R/T vector as well as ECOR1 and HindIII restriction enzymes were used to confirm the clone. Sample was sent to Macrogen Korea for sequencing in sense and antisense direction and final sequence was submitted in the public database of National Center for Biotechnology Information (NCBI) Molecular Evolutionary Genetics Analysis (MEGA) version 6.0 software was used to construct a neighbor joining tree with other reported sequences with Acc. No. LT592134 (Tamura *et al.*, 2013).

RESULTS AND DISCUSSION

In this study total fifteen different DNA samples were cloned and sequenced through (Sanger method). Amplified PCR product size was 1500bp (Fig. 2a). After that cloning of the PCR product was done by using standard protocol for cloning in PTZ57R/T vector. Restriction analysis for the confirmation of clone product was done by using ECOR1 and HindIII enzymes (Fig. 2b).

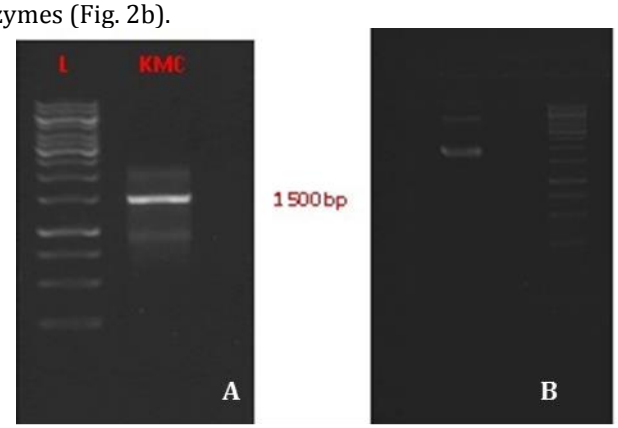

Figure 2: Gel electrophoresis of amplified PCR product (a) and restriction analysis (b) for the confirmation of clone.

Witches' broom disease of lime (WBDL), associated with 'Candidatus Phytoplasma aurantifolia' has been reported as a major cause of lemon trees decline in Mexico, Oman, United Arab Emirates,Iran and India (Mardi *et al.*, 2011). Infected lime trees showed number of Witches brooms bearing small new pale green leaves in earlier stages while older leaves dry up and eventually fall away leaving behind dead twigs, shoots and dried witches' broom. The normal host range of 'Ca. P. aurantifolia' includes Citrus aurantifolia, C. medica, C. limetta, C. lemon and C. jambhiri. Phytoplasmas have been detected in HLBinfected trees of citrus in Brazil, that is seriously threatening global citrus production (Teixeira *et al.*, 2008; Chen *et al.*, 2009). The pathogenic role of phytoplasmas was not clearly elucidated, but they can be detected in symptomatic plants in cases in which HLB is not detected (Bertaccini *et al.*, 2014). Our study exactly related to this study as symptoms were found in leaf sample of HLB infected trees of citrus.

Our sequence showed maximum 97% similarity in BLAST with this GenBank acc. No. KF101620 which is 1311bp long and isolated from Homosapiens skin from USA in 2015. While during pair wise sequence distance it showed maximum 45.6% similarity with Azomonas agilis (NR_041034). According to Phylogenetic tree (Fig. 3) analysis this uncultured bacterium (Acc. No.LT592134) lies in separate clade in between Kurthia sp. and Arthobacter sp. This

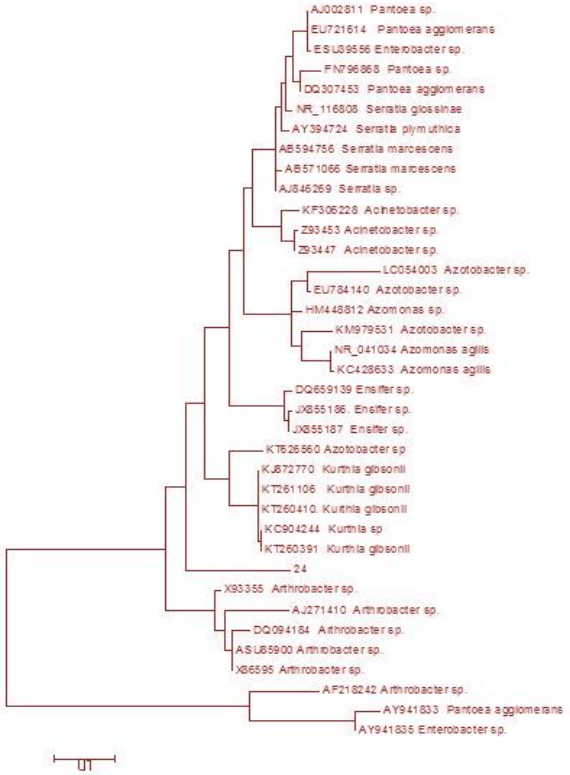

Figure 3: Phylogenetic analysis of cloned sequence with previously reported sequences.

bacterium could be isolate of Kurthia sp. or Arthobacter sp. To our knowledge this might be new uncultured bacterium that is not reported yet in Pakistan.

CONCLUSION

The uncultured bacterium which was found this study could be a novel strain of Kurthia and Arthobacter that still needs characterization with specific primers of these suspected bacteria.so it is a new report of the presence of uncultured bacterium in HLB infected leaf sample of Mandarin from Punjab Pakistan.

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