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CHARACTERIZATION OF PHYTOPLASMA ASSOCIATED WITH PERIWINKLE WITCHES'-BROOM DISEASE IN PAKISTAN

Rizwan Khan[1], Najeeb Ullah[2], Khalid Pervaiz Akhtar[2], Shabir Ullah[3], Ijaz Ahmad[1], Muhammad Fahim[4]
[1] The University of Agriculture Peshawar, Pakistan.
[2] Nuclear Institute for Agriculture and Biology, Faisalabad, Pakistan.
[3] Amir Muhammad Khan Campus (Mardan), The University of Agriculture, Peshawar, Pakistan.
[4] Centre for Omic Sciences, Islamia College University Peshawar, Pakistan.

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ABSTRACT

Severely malformed periwinkle plants suspected of phytoplasma infection were observed in the lawn at Institute of Biotechnology and Genetic Engineering (IBGE), the University of Agriculture, Peshawar, Pakistan. Approximately 10 to 25% of the plants were infected and based on nested polymerase chain reaction (PCR) results using phytoplasma-specific primer pairs (P1/P7 followed by R16F2n/R16R2), phytoplasma was detected in these plants. One amplicon of nested PCR was subsequently sequenced (GenBank accession No. MH396693) which shown 99% identity with 16Sr-I subgroup phytoplasma in BLASTn analysis and was also grouped together with phytoplasmas of subgroup16Sr-IB in the phylogenetic tree. To our knowledge, this is the first record of 16Sr-IB phytoplasma infecting periwinkle plants in Khyber Pakhtunkhwa province of Pakistan.

Corresponding Author: Muhammad Fahim
Email: fahim@icp.edu.pk

INTRODUCTION

Phytoplasmas are phloem inhabiting obligate parasites known to cause economical diseases in numerous plant species worldwide characterized by phyllody, virescence and witches' broom symptoms (Win and Jung, 2012). Presently, in Pakistan phytoplasma causing phyllody disease is an emerging problem in many crops (pulses, sesame, and tomato) and is known to be transmitted by an insect vector, leafhopper (*Orosius albicinctus*). Phytoplasmas can infect several weed species which can serve as inoculum sources for other important commercial crops and recently a weed is first time identified as a natural host of 16SrII-D phytoplasma in Pakistan (Akhtar et al., 2018). Phytoplasma of the same group can induce different symptoms depending on the host plant. Periwinkle (*Catharanthus roseus*) is an important floral species cultivated ornamentally and as a medicinal plant in many parts of the world. It possess useful alkaloids having medicinal properties (Nejat et al., 2015). However, periwinkle plants are known to be susceptible to different groups of phytoplasma and mostly manifest symptoms of virescence and proliferation. Similar symptoms of virescence and proliferation were observed in periwinkle plants grown in the lawn at Institute of Biotechnology and Genetic Engineering (IBGE), the University of Agriculture, Peshawar, Pakistan. Therefore, this study was conducted to find and characterize at the molecular level the phytoplasma associated with the symptomatic periwinkle plants observed.

MATERIALS AND METHODS

In October 2017, Periwinkle plants gown in the lawn at IBGE, the University of Agriculture, Peshawar, Pakistan were observed showing yellowing, virescence and Witches' broom symptoms reminiscent of phytoplasma infection. Approximately 10 to 25% of the plants were found infected. To check these observed plants for phytoplasma infection, leaf samples were collected from 10 symptomatic and four asymptomatic periwinkle plants. Polymerase chain reaction (PCR) techniques are regarded as the method of choice for the detection of phytoplasma. Therefore, total DNA was extracted from these leaf samples using a CTAB-based DNA extraction method (Doyle and Doyle, 1990) and its quality was checked on 1% agarose gel. Also, DNA concentration and quantity was measured using NanoDrop (Thermo-Scientific NanoDrop 2000c Spectrophotometer, USA). To confirm presence/ absence of phytoplasma, nested polymerase chain reaction (PCR) using universal phytoplasma primers P1/P7 (Schneider et al., 1995) followed by R16F2n/R16R2 (Lee et al., 1993) was carried out to amplify 16S rRNA gene from 10 diseased and four healthy periwinkle plants. PCR mixture (25 µl) containing 10.5 µl nuclease free water, 12.5µl of 2x DreamTaq Green PCR Master Mix (Thermo Scientific), 1µl of DNA and 0.5µl (5pmol/ul) of each primer were used. Both direct and nested PCR were performed for 35 cycles after initial denaturation (94°C for 5 min). For direct PCR with P1/P7 primers cycling conditions were as follow: denaturation for 1 min at 94°C, annealing for 1 min at 54°C and extension for 2 min at 72°C. While for nested PCR with primers R16F2n / R16R2 condition were set as follow: denaturation for 1 min at 94°C, annealing for 1 min at 50°C and extension for 1.5 min at 72°C. The final extension was performed for 10 min at 72 °C. Amplified 16S rDNA fragment of one representative sample gel was purified and directly sequenced in both directions with R16F2n/R16R2 primers. The sequences were analyzed using BioEdit software, deposited in NCBI GenBank whereas reference sequences were obtained from GenBank (http://www.ncbi.nlm.nih.gov/genbank/). The phylogenetic tree was constructed using MEGA-7 (Kumar et al., 2016) with 1,000 pseudoreplicates.

RESULTS AND DISCUSSION

The periwinkle plants grown at IBGE were infected by phytoplasma showing characteristic symptoms of virescence (Figure 1), yellowing, small and deformed leaves at the tip of shoots and witches' broom (Figure 2). Phytoplasma presence in these plants was confirmed using PCR assay. Indirect PCR amplicons of ~1.8 was amplified using primers P1/P7 confirming the association of phytoplasma with symptomatic periwinkle plants, whereas no PCR amplified product was obtained from four asymptomatic periwinkle plants sample. In the nested-PCR typical product of ~1.25 kbp in size were successfully amplified using R16F2n/R16R2 primers. Amplified 16S rDNA fragment of ~1.25 kbp size of one representative sample was directly sequenced and the obtained sequence was BLASTn analyzed to compare with reference sequences of phytoplasmas in GenBank database.

Figure 1. Phytoplasma infected periwinkle plant showing virescence symptoms

Figure 2. Phytoplasma infected periwinkle plant showing yellowing and Witches' broom symptoms

The obtained sequence showed 99% homology with 16S rDNA from several phytoplasmas related to '*Candidatus* Phytoplasma asteris' of group 16Sr-I and the consensus sequence was submitted to GenBank (Accession No. MH396693). Phylogenetic analysis using the neighbor-joining method (MEGA7) also grouped our characterized phytoplasma (periwinkle-Pak; accession No. MH396693) with phytoplasmas of subgroup16Sr-IB (Figure 3).

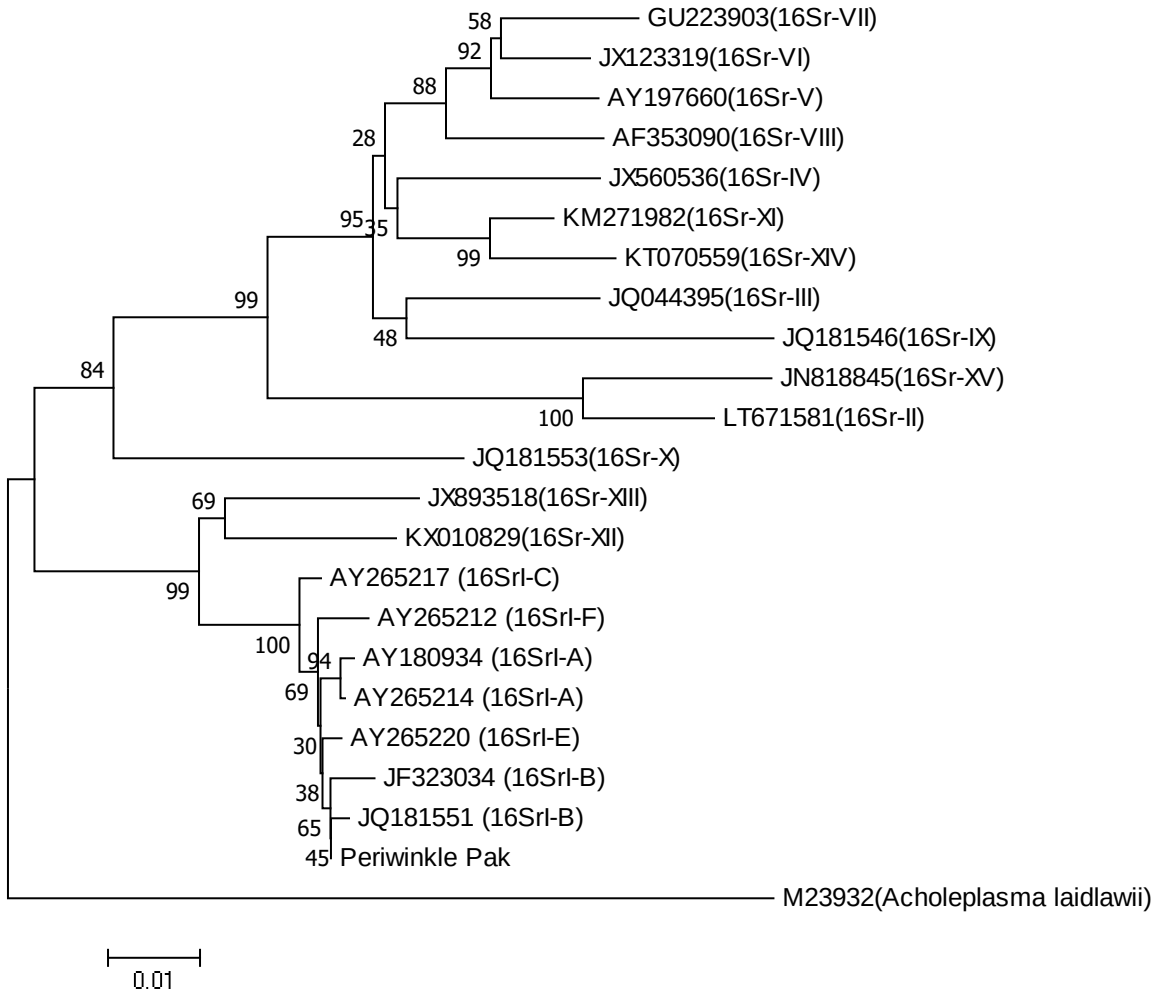

Figure 3: Phylogenetic tree based on 16S rRNA sequences showing the relationships of Periwinkle phytoplasma from Pakistan (Periwinkle Pak) with reference 16Sr phytoplasma groups while *Acholeplasma laidlawii* (M23932) was used to root the tree. GenBank accession numbers are given and phytoplasmas subgroups sequences are shown in parenthesis. Numbers on the branches are bootstrap (confidence) values of 1000 replicates. The bar indicates the number of substitutions per nucleotide position. The evolutionary distances were computed using the Kimura 2-parameter model using MEGA7.

As phytoplasmas are obligate parasite, fulfillment of Koch's postulates is technically challenging and has not been achieved (Verdin et al., 2003). However, in our study the presence of phytoplasma only in infected (symptomatic) periwinkle plants by nested-PCR is the confirmation that the causal agent of the periwinkle disease is phytoplasma. This was further supported by sequencing the 16S rDNA gene of one representative sample. Previously, 16Sr-I phytoplasma was reported in periwinkle plant grown in Islamabad city of Pakistan (Fahmeed et al., 2009); however, to our knowledge, this is the first report of '*Candidatus* Phytoplasma asteris' (16Sr-IB) infecting periwinkle plants in Khyber Pakhtunkhwa Province of Pakistan. From this study, it is

concluded that phytoplasma infecting Periwinkle in Pakistan belongs to 16Sr-IB. Furthermore, high susceptibility of periwinkle and its perennial nature can play a major role in phytoplasma survival in the region and its insect vector and more importantly, will serve as a potential inoculum source. It also enhance the probability of phytoplasma infection in other crop grown in the area which needs to be explored to prevent possible outbreaks of phytoplasma diseases.

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