

Journal of Scientific Research and Reports

Volume 30, Issue 12, Page 537-547, 2024; Article no.JSRR.128060 ISSN: 2320-0227

## Identification and Molecular Characterization of a 16SrV-F Phytoplasma Associated with Little Leaf and Witches' Broom Disease in Jackal Jujube (*Ziziphus oenoplia* L.) in Kerala, India

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### Authors' contributions

This work was carried out in collaboration among all authors. Author MR surveyed and investigated the work, conceived the idea, did microscopic analysis, and drafted the original manuscript. Author SK did funding acquisition, and project administration, searched for resources, supervised the study, did data validation, reviewed & edited the manuscript. Author JMJ performed DNA extraction PCR and assisted in molecular analysis. Author MKS did molecular phylogeny analysis and interpretation, validation of data, reviewed and edited the manuscript. Authors JUK and DJ did data validation, reviewed and edited the manuscript. All authors read and approved the final manuscript.

### Article Information

DOI: https://doi.org/10.9734/jsrr/2024/v30i122698

#### **Open Peer Review History:**

This journal follows the Advanced Open Peer Review policy. Identity of the Reviewers, Editor(s) and additional Reviewers, peer review comments, different versions of the manuscript, comments of the editors, etc are available here: https://www.sdiarticle5.com/review-history/128060

> Received: 11/10/2024 Accepted: 13/12/2024 Published: 20/12/2024

**Original Research Article** 

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*Cite as:* R., Murali, Shambhu Kumar, Jithu U. Krishnan, Donald James, Mahadeva Kumar S, and Jain Mary Jose. 2024. "Identification and Molecular Characterization of a 16SrV-F Phytoplasma Associated With Little Leaf and Witches' Broom Disease in Jackal Jujube (Ziziphus Oenoplia L.) in Kerala, India". Journal of Scientific Research and Reports 30 (12):537-47. https://doi.org/10.9734/jsrr/2024/v30i122698.

### ABSTRACT

Ziziphus oenoplia (Jackal jujube), a perennial thorny shrub in the Rhamnaceae family, is widely distributed in India, particularly in Kerala, Karnataka, Andra Pradesh, Telangana, and Maharashtra, specifically in the Western Ghats, renowned for its medicinal properties, attributed to alkaloids known as ziziphines, the plant is crucial in Ayurvedic medicine. Despite its medicinal importance, Z. oenoplia faces susceptibility to little leaf and witches' broom diseases incited by phytoplasma, suspected as potentially playing a role in sandal spike disease epidemics as an alternate host in Kerala. Plant samples, both infected and healthy, were collected from Wadakkancherry forest area and the Kerala Forest Research Institute campus. The pathogenic agent responsible for wild jujube little leaf and witches' broom disease was identified through symptomatology, microscopic observation (Scanning Electron Microscope), PCR, nested PCR, and phylogenetic analysis. Symptomatic features included a severe reduction of leaf size, yellowing, and numerous small leaves which appeared in clusters resembling witches' broom. Scanning electron microscopic (SEM) assay confirmed the presence of phytoplasma bodies, which are pleomorphic in shape and absence of cell walls, were found in the phloem sieve tubes of diseased Ziziphus plants, but absent in healthy Ziziphus plants. Molecular analysis using PCR and nested PCR primers authenticated the existence of phytoplasma in the diseased plants. Results of the nucleotide sequence analysis of 16s ribosomal gene showed 100% nucleotide identity with 16SrII phytoplasma isolates, specifically Candidatus Phytoplasma balanitae isolate JWB K15 (MN902087). The study underscores the molecular distinction between the phytoplasma strains infecting Ziziphus and Sandalwood, providing valuable insights for disease management and ecological preservation.

Keywords: Plant diseases; phytopathogens; Ziziphus; phytoplasma; phylogeny.

### 1. INTRODUCTION

Jackal jujube (Ziziphus oenoplia L.) Mill. also known as wild jujube, small fruited jujube belongs to the family Rhamnaceae, is an incessant flowering, and thorny shrub mainly found in the deciduous forests types of southern part of the country. In India, it is distributed in Kerala, Karnataka, Andra Pradesh, Telangana and Maharashtra and specifically in Western Ghats. Z. oenoplia bears edible fruits and several parts of this plant are commonly used for treatment in Avurvedic medicine (Eswari et al., 2014). This plant contains ziziphines alkaloids belong to the cyclopeptide alkaloids group, which has old times of usage in Ayurveda medicine or herbal medicine. The various plant parts are mainly used for the treatment of hyperacidity, digestive ailments, asthma, stomach aches, ulcer, obesity, diuretic conditions and also as an antiseptic, stringent, and wound healing properties agent (Shukla et al., 2016). However, this plant is not grown or cultivated commercially, but, it is an important and significant plant species due to its great medicinal properties, biodiversity aspects and ecological point of view. Despite of its much medicinal importance, this species is faces highly susceptibility to the diseases called little leaf and witches' broom caused by phytoplasma. Severe incidence of little leaf and witches' broom diseases on Z. oenoplia was noticed in natural forests in Kerala. Researchers suspected that, this host have a possible role in the epidemics of spike disease of Sandalwood as an alternate host in Kerala (Ghosh et al., 1985).

Phytoplasma belongs to the class of mollicutes formerly known as mycoplasma-like organisms (MLO), is an economically important and large group of plant pathogenic bacteria. These pathogens lack cell walls, bounded by triple layer unit membrane, pleomorphic in shape and specially reside intracellularly in haemocyl of insect vectors and plant phloem tissues. Phytoplasma are unable to culture under in vitro artificial medium, hence these are assigned as "Candidatus" status based on 16S ribosomal DNA gene sequence and Restriction Fragment length analysis (Lee et al., 1993; Lee et al., 200 Lee et al., 1993, Wang et al., 2018). Phytopathogenic phytoplasmas were first discovered and identified by Doi et al. (1967) in Mulberry yellow dwarf disease in Japan. These pathogens are mainly transmitted from infected plant to healthy plant by different insect vectors which are phloem feeding in nature such as leafhoppers, plant hoppers and psyllids, dodder, and artificial transmission by means of different grafting methods (Weintraub & Beanland, 2006; Murali et al., 2019). Phytoplasmas are known to infect more than 700 important plant species severely belonging to cereals, pulses, oil seeds,

vegetables, fruits, palms, ornamentals, and forest plants worldwide (Rao, 2021). The impact of diseases caused by phytoplasma on forest ecosystems can be significant, as they can cause complete mortality and reduced productivity of important tree species. This can have economic and ecological consequences, such as reduced timber production and changes in ecosystem structure and forest biodiversity (Marcone et al., 2018; Marcone et al., 2021).

The plants infected with phytoplasma can display different kinds of distinct symptoms on many crops (Bertaccini et al., 2014; Omar, 2017; Rao et al., 2017; Sundararaj et al., 2021).

Detection and diagnosis of phytoplasmas are done mainly through the use of both traditional and molecular (Polymerase Chain Reaction) methods which targeting the amplification of 16S ribosomal encoding genes, and this used as one of the criteria for the classification of phytoplasma into different taxonomic groups (Lee et al., 2000). As of now, on the 16S rRNA encoding gene sequence and Restriction Fragment length analysis, more than 34 main 100 subgroups groups and of diverse phytoplasmas were identified across the world (Bertaccini & Lee, 2018; Kumari et al., 2019). Hence, the aim of the investigation was to detect and characterise little leaf and witches' broom diseases of Ziziphus oenoplia L. in Kerala at molecular level.

### 2. MATERIALS AND METHODS

### 2.1 Disease Incidence, Infected Sample Collection, Symptomatological and Morphological Identification

The infected and healthy samples of Jackal jujube plants which showing the typical symptoms of little leaf and witches' broom were collected from different locations of Wadakkanchery natural forest from Kerala (10.71232° N latitude, 76.26483° E longitude) (Fig. 1). The collected infected and healthy samples were brought to the Forest Pathology Laboratory of Kerala Forest Research Institute campus, Kerala India for proper pathological investigation. The photographs of diseased host plant, infected leaves showing the symptoms, and healthy plants were taken using Sony Cybershot (Model: DSC-W810/B) digital camera during the time of collection of samples. The detailed symptomatological characterization was carried out based on the visualization of

appearance external symptoms in the form of severe reduction in leaf length and size. vellowing of leaves, huge number of small leaves appeared in cluster which resembling whiches' broom. Scanning electron microscopic analysis was carried out using TESCAN VEGA 3-LMU and analysied using TESCAN software in college of veterinary and animal science Mannuthy, Thrissur, Kerala to know the presence of morphological phytoplasma and their characterization. For preparation of SEM analysis, cross sectioning of stem was done for infected and healthy samples for comparison. After sectioning the samples were sputtercoated for conductive properties. Conductive coating was done with Para film and samples were dehydrated by the application of alcohol. After the preparation, the samples were placed in the vacuum environment of the microscope. Different magnifications were used for capturing the images.

### 2.2 Molecular Identification

For confirmation of molecular identity of pathogens, total genomic DNA from the phytoplasma infected diseased samples was extracted using a modified Phenol: Chloroform method (CTAB) Stange et al. (1998). For this, 150–200 mg of infected samples along with healthy were ground and powdered using 20 ml of liquid nitrogen in separate pestle and mortar for infected and healthy samples. Around 2–2.5 ml CTAB DNA extraction buffer was added to the sample and ground thoroughly.

Approximately 750 microliters of ground sap was loaded into 1.5 ml micro centrifuge tube and incubated in water bath (LABLINE) at temperature of 65°C for the period of 30 minutes. After incubation, 750 microliters of mixture of Chloroform: Isoamyl alcohol (24:1 concentration) was added to the samples and mixing was done to form an emulsion by inverting the tubes. The mixture was subjected to centrifugation (CRYOZEN 1730R) at 13,000 rpm for the period of 10 minutes. The supernatant was collected and thoroughly mixed with 300 microliters of Isopropanol and kept for overnight incubation at -20°C temperature. The contents of the incubated mixture were centrifuged at 13000 rpm for 10 minutes; the supernatant was removed by retaining the pellet. Later pellet was washed with 500 microliters of 70 per cent ethanol and centrifuged at 14000 rpm for 5 minutes. After centrifugation, ethanol was removed and pellet was dried in a vacuum drier (make) for five

minutes. In final step, dried pellet was dissolved in TE buffer (1X) depending upon the quantity of DNA extracted and stored at  $-20^{\circ}$ C temperature. The quality and concentration of extracted DNA was tested using Nanodrop (Thermo Scientific, Model: ND–One–W) and the values are 678 mg/ µl, 2.14 at 260/280 for infected sample and 337 mg/µl, 1.83 at 260/280 for healthy sample.

Extracted DNA from the infected and healthy samples was subjected for PCR amplification using phytoplasma specific universal primers P1 (Deng and Haruki, 1991) and Primer P7 (Smart et al., 1996) specific to the 16S ribosomal gene. The details of the primers used in this study was given in Table 1.

The PCR reaction was executed with a thermal cycler (Bio–Rad T100, India). Direct PCR conditions were denaturation at 94°C for 3 min, followed by 30 cycles of 94°C for 1 min, 53°C for 2 min and 72°C for 2 min and a final extension for 5 min at 72°C (Kirkpatrick et al., 1994; Murali, 2012). Purified PCR products were subjected to an ethidium bromide–stained 1 per cent agarose gel electrophoresis along with a DNA ladder to quantify the size of the amplified DNA. Amplified

PCR products were further purified and subjected to nested PCR amplification using nested primers pair R16F2n and R16R2 (Lee et al., 1993) to amplify the unamplified and conserved regions of the genome. The in were: conditions used nested PCR denaturation at 94°C for 5 min, followed by 30 cycles of 94°C for 50 sec, 55°C for 45 sec and 72°C for 1.5 min and final extension for 7 min at 72°C. After nested PCR, the products were subjected to an ethidium bromide-stained 1 per cent agarose gel electrophoresis along with a DNA ladder to quantify the size of the amplified DNA (Gao et al., 2011, Gundersen and Lee, 1996). The purified nested PCR products were subjected to sequencing of conserved region of 16Sr ribosomal gene at GeneSpec pvt. Ltd., Cochin to know the genetic similarities. The positive amplicons of PCR were sequenced bidirectionally and gathered using BioEdit software (Hall, 1999). The CLUSTAL W approach from the MEGA X programme was employed to align 16S rRNA sequence with similar and closely related sequences already present in NCBI databases. The obtained sequence is submitted as KFRI-P001 to NCBI gene bank database with the accession number (PP159097) for further reference.



Fig. 1. The study area map

### Table 1. Details of the primers used in direct PCR and Nested PCR in the detection of Phytoplasma 16S ribosomal gene

	Name of the Primer	Nucleotide sequence	References
Direct PCR primers	P1 (Forward Primer)	5'AAGAGTTTGATCCTGGCTCAGGATT 3'	Deng and Hiruki (1991)
	P7 (Reverse Primer)	5'CGTCCTTCATCGGCTCTT 3'	Smart et al. (1996)
Nested PCR primers	R16F2n (Forward Primer)	5'ACGACTGCTGCTAAGACTGG 3'	_ Lee et al. (1993)
	R16R2 (Reverse Primer)	5'TGACGGGCGGTGTGTACAAACCCCG 3'	

### 2.3 Phylogenetic Analysis

The consensus sequences KFRI–P001 closely related strains were obtained and assembled. The results garnered from the nucleotide BLAST (BLASTn) tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi) of NCBI database were used to create the phylogenetic tree using Maximum Likelihood method (ML). The sequences which generated were aligned using MEGA X software to perform phylogenetic sequence analysis (Kumar et al. 2018). The details of sequence from the present investigation and reference sequences were indicated in the phylogenetic tree, as well as the respective phytoplasma strains details.

### 3. RESULTS AND DISCUSSION

The clear symptoms of phytoplasma infection were observed in the form of severe reduced leaf size and length, yellowing, huge number of small leaves appeared in cluster which resembling witches' broom compared to healthy plants (Fig. 2. A,B). The similar symptoms were observed by Venkataravanappa et al (2023).

The Jackal jujube plants infected by phytoplasma were tall, weak, and infected leaves which are small in size and yellow in appearance. The axillary shoots or branches are proliferated which produce many small secondary branches covered with little yellow leaves resembling a cluster or bushy appearance of the infected plants. They also reported that, the disease incidence was varied from 50-60 per cent. Pai et al., (2019) observed jujube (Ziziphus oenoplia L.) severe plants exhibited witches' broom symptoms during survey conducted on October-November 2019 in Karnataka state of India. Khan et al., (2008) reported the presence of 'Candidatus Phytoplasma ziziphi' strains in two different species of Jujube trees (Ziziphus spp.) in Uttar Pradesh, India, the symptoms includes resetting of leaves, proliferation of axillary branches, little leaves and witches' broom-like appearance.

The abnormal symptoms development due to the phytoplasma induced esoteric disturbances in the concentration of growth regulators or plant hormones in the host plant (Das and Mitra, 1998; Murali et al., 2023). Some molecular evidences also clearly showed the development of typical abnormal symptoms of phytoplasma infection due the imbalance of plant growth regulating hormones. Further, this hormonal imbalances is mainly due the high utilization of carbon sources, which results in high growth rates, excessive cell multiplication, production of severe and abnormal type of symptoms in the form of yellowing, dwarfism, and decline in Onion yellows disease (Namba, 2019).

scanning electron microscopic (SEM) The analysis confirmed the presence of phytoplasma pleomorphic bodies (400-1600 nm) lacking cell wall were noticed in the phloem sieve tubes of stem sections from diseased Ziziphus plant and not found in healthy plants (Fig. 2C,D). Naik et al. (2023) examined the crown choking infected Arecanut samples through transmission electronic microscopic assay with the help of Sri Sai Histology Centre lab, Hyderabad. The thin sections of the leaf midrib from diseased Arecanut plant were done and subjected to microscopic analysis. Microscopic assay revealed the presence of pleomorphic bodies with absence of cell wall were observed in the phloem sieve tube confirmed the phytoplasma infection. The sizes of the phytoplasma cells were ranging from 500 - 2000 nm. Phytoplasmas are gram positive bacteria with high guanine and cytokine content, appear as pleomorphic shape as complex of multi branched, spheroidal or filamentous bodies. The size of the phytoplasma cells ranging from 175-400 nm in diameter for the oblong and spherical cells and up to 1700 nm long for the filamentous types (Murali, et al.,

2023). The transmission of phytoplasmas in natural condition mainly through the phloem feeding insect vectors, these insects deposit the phytoplasma cells in the phloem tissues while feeding and phytoplasma requires high sterol content for their growth, hence these specifically colonize the sieve tubes.

In direct PCR amplification by using universal phytoplasma detection primer pair P1 and P7 specific to the 16S rRNA gene of phytoplasma vields about 1.8 kb products and nested PCR amplifies 1.3kb (Fig. 3) of product using nested PCR primer pair R16F2n and R16R2 from the infected Ziziphus plants but not from the healthy plants (Fig. 4). Venkataravanappa et al (2023) isolated DNA from 3 symptomless and 16 symptomatic Jackal Jujube plants collected from different locations of Karnataka, India. The extracted genomic DNA was subjected to direct PCR amplification specific to the 16S ribosomal encoding gene of phytoplasma using universal primer pair P1/P7 along with positive controls (Sesame phyllody and Brinjal little leaf

phytoplasma) and expected amplicon size of 1.8 kb was obtained in direct PCR. The amplified PCR product was further analysed in nested PCR using nested PCR primers pair R16F2n/R2. R16mF2/16mR1, and fU5/rU3 especially for the amplification of unamplified genomic segments present within 16S rRNA encoding gene of phytoplasma (Gundersen & Lee, 1996; Lee et al., 1998). Nested PCR yields an amplicons size of 1.2 kb, 1.4 kb, and 0.8 kb, respectively were obtained. Pai et al., (2019) pathologically investigated Ziziphus oenoplia plants showing witches' broom symptoms, to verify the presence of phytoplasma. Total genomic DNA extracted from infected leaves of Jujube plants using a modified phenol chloroform method (CTAB) and PCR amplification was carried out using P1/P7 universal phytoplasma primer pairs and nested PCR using primers 3Far/3Rev specific to the 16S rRNA gene of phytoplasma. The amplicons of size 1.3 kb of were perpetually amplified from all the diseased plants in nested PCR detection, on other hand, no amplification was found in asymptomatic plant samples.



Fig. 2. Symptoms on Ziziphus oenoplea in wild A. Severe incidence of little leaf and witches' broom disease on Ziziphus oenoplea B. Healthy twig with leaves C. Little leaf and witches' broom symptoms D. SEM image of Phloem cells of healthy Ziziphus plant E. SEM image of Phloem cells of little leaf and witches' broom infected Ziziphus plant (Presence of phytoplasma cells in the phloem tissue)

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Fig. 3. Detection of phytoplasma in little leaf and witches broom infected Ziziphus plant samples through direct PCR using P1and P7 primers



Fig. 4. Detection of phytoplasma in little leaf and witches broom infected Ziziphus plant samples through nested PCR using R16F2n and R16R2 primers

Cloning and molecular sequence analysis showed the highest nucleotide identity (100%) with Candidatus Phytoplasma balanitae isolate JWB K15 (MN902087) which belongs to 16srV-F group. Where, the sandal spike phytoplasma strain belongs to 16Srl-B. Hence the two phytoplasma strains infecting Ziziphus oenoplia and Sandalwood are different at molecular level. Bertaccini et al., (2022) given the revised guidelines for taxonomy and naming of new strains of phytoplasma with 98.65% threshold of 16SrRNA gene. Snehi et al (2020) reported the natural occurrence of little leaf and witches' broom diseases of Z. oenoplia from Barkatullah University campus, Bhopal. Phytoplasma 16Sr ribosomal gene was detected by direct PCR

using phytoplasma specific primers P1/P6, amplified DNA diluted in 10:1 concentration and subjected to nested PCR amplification. Nested PCR yields about 1.2 kb of product and sent for Nucleotide sequence data were sequencing. analysed in NCBI BLASTn. BLASTn analysis of 16S rRNA gene revealed that, isolates from this study MK975463 and MK975462 showed highest (99%) similarity with Jujube witches'-broom phytoplasma strains MH972556, MH972553, and MH972548 of Ziziphus sap sucking insects from India and Candidatus Phytoplama balanitae strain (HG937644, LT558785, MH819290) of Z. mauritiana and Z. oenoplia belongs to Elm vellows group phytoplasma (16SrV) from India.



# Fig. 5. Phylogenetic tree generated from Maximum–Likelihood method (ML) with selected phytoplasma 16S rRNA gene sequences. The sequence of the phytoplasma from this study is marked in red

The phylogenetic analysis of 16S ribosomal gene sequence of the phytoplasma strain inciting Z. oenoplia witches' broom and little leaf using Maximum Likelihood method indicates its close relationship with 'Candidatus Phytoplasma balanitae' strains and belongs to 16SrV-F subaroup phytoplasma (Fig. 5). The R16F2n/R16R2 sequence of the phytoplasma strain causing crown choking of Arecanut palm was analysed in NCBI BLASTn and results revealed that, highest similarity (99.92%) related 16Srll group, Candidatus Phytoplasma to aurantifolia (MT555412, MT555411) which were identified Croton bonplandianus in in India (Naik et al., 2023). Maharashtra, Characterization of phytoplasmas was described 16S ribosomal gene sequence and on Restriction Fragment Length Polymorphism. The 16Sr RNA gene is present in all the prokaryotes and its conserved and variable regions make it convenient for phylogenetic and taxonomic classification and making into different groups.

### 4. CONCLUSION

In conclusion, Jackal jujube is a perennial, flowering shrub has medicinal benefits faces high susceptibility to little leaf and witches' broom diseases. Phytoplasma associated with little leaf and witches' broom disease of Jackal jujube was identified and confirmed on the basis of symptoms, SEM micrographic observations and molecular phylogenetic analysis as high similarity to the starin Ca. P. balanitae which belongs to the 16SrV-F group. Hence, based on literature survey, this is first report of little leaf and witches' broom phytoplasma diseases on Jackal jujube from Kerala state of India. There is still scanty information about natural insect vectors which Ziziphus transmits the witches' broom phytoplasma from infected to healthy plant. Therefore, field and lab oriented interventions are much required to address the unanswered questions regarding phytoplasma host range, different strain virulence, insect vectors. pathogenicity, phytoplasma-plant interactions,

host resistance and epidemiological factors. Knowledge of these features is the basis for the development of suitable disease management practices. The results of this research may help the formulation of effective control measures and management strategies aimed at safeguarding plants from the impact of this phytoplasma pathogen in India. It is advisable to conduct additional studies for disease surveillance, vectors involved in transmission and protection to mitigate the occurrence of the disease.

#### **DISCLAIMER (ARTIFICIAL INTELLIGENCE)**

Author(s) hereby declare that NO generative Al technologies such as Large Language Models (ChatGPT, COPILOT, etc.) and text-to-image generators have been used during the writing or editing of this manuscript.

### ACKNOWLEDGEMENTS

The authors would like to thank the Director, KSCSTE–Kerala Forest Research Institute, Kerala for providing all necessary facilities and financial support under the project (PG RP 862/2023). Authors are also thankful to the Department of Biotechnology (DBT), Government of India project (BT/PR29521/FCB/ 125/15/2018) under which the molecular facility was utilised for this study.

### **COMPETING INTERESTS**

Authors have declared that no competing interests exist.

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