Molecular detection and phylogenetic characterization of little leaf and witches' broom diseases associated with Jackal jujube (*Ziziphus oenoplia* L.) caused by phytoplasma 16Sr-VF strain in Kerala, India

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 diseasesincited 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) assayconfirmedthe 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, smallfruited jujubebelongs to the family Rhamnaceae, is an incessantflowering, and thorny shrub mainly found in the deciduous foreststypes 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 andseveral parts of thisplant are commonly used for treatment in Ayurvedic 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 andalso asanantiseptic, stringent, and wound healing properties agent (Shukla*et al.,* 2016).However, this plant is not grown or cultivatedcommercially, 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' broomcaused by phytoplasma. Severe incidence of little leaf and witches' broom diseases on *Z. oenoplia* wasnoticedin 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 haemocylof 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.,* 200Lee *et al.,* 1993). Phytopathogenic phytoplasmas were first discovered and identified by Doi *et al.* (1967) in Mulberry yellow dwarf disease in Japan. These pathogens aremainly transmitted from infected plant to healthy plantby differentinsect vectors which are phloemfeeding 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 displaydifferent 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 rRNAencoding gene sequence and Restriction Fragment length analysis, more than 34 main groups and 100 subgroups 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. Material 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 andsize, yellowing 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 phytoplasma and their morphological characterization. For preparation of SEM analysis, cross sectioning of stem was done for infectedand healthy samples for comparison. After sectioning the samples were sputter–coated 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 sampleswas 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 groundthoroughly.

Approximately 750 microliters of ground sap was loaded into 1.5 ml micro centrifuge tube and incubated in water bath(LABLINE)attemperature of 65^{o}C for the period of 30 minutes. After incubation, 750 microlitersof 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 microlitersof Isopropanol and kept for overnight incubation at –20^{o}C temperature. The contents of the incubated mixture were centrifuged at 13000 rpm for 10 minutes; the supernatant was removedby retaining the pellet. Later pellet was washed with 500 microlitersof 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^{o}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 no.1

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	Reference
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	R16F2 (Forward Primer)	**5'**ACGACTGCTGCTAAGACTGG**3'**	Lee *et al*. (1993)
	R16R2 (Reverse Primer)	**5'**TGACGGGCGGTGTGTACAAACCCCG**3'**	

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 conditions used in nested PCR were: 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 GeneSpecpvt. 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.

2.3. Phylogenetic analysis

The consensus sequences KFRI–P001closely 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 MaximumLikelihood method (ML). The sequences which generatedwere 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 wereindicated 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. 2A,B).** The similar symptoms were observed by Venkataravanappa *et al* (2023).

The Jackal jujube plants infected by phytoplasma were tall, weak, and infected leaveswhich 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 bushyappearance 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.) plants exhibited severe 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 imbalancesis mainly due the high utilization of carbon sources,which results in high growth rates, excessive cell multiplication, production of severe and abnormaltype of symptomsin the form of yellowing, dwarfism, and decline in Onion yellows disease (Namba, 2019).

The scanning electron microscopic (SEM) 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 assayrevealed 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 yields 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 infectedZiziphus plants but not from the healthy plants**(Fig. 4).**Venkataravanappa *et al* (2023) isolatedDNA 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 pairP1/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 rRNAencoding 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.

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 16SrI–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 sequencing. Nucleotide sequence data were 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 Phytoplamabalanitae* strain (HG937644, LT558785, MH819290) of *Z. mauritiana*and *Z. oenoplia* belongs to Elm yellows group phytoplasma (16SrV) from India.

The phylogenetic analysis of 16S ribosomal gene sequence of the phytoplasma strain inciting Z. *oenoplia* witches' broom and little leaf using MaximumLikelihood method indicates its close relationship with '*Candidatus Phytoplasma balanitae*' strains and belongs to 16SrV–F subgroup phytoplasma**(Fig. 5).** The R16F2n/R16R2 sequence of the phytoplasma strain causing crown choking of Arecanut palm was analysed in NCBIBLASTn and results revealed that, highest similarity (99.92%) related to 16SrII group, *Candidatus Phytoplasma aurantifolia* (MT555412, MT555411) which were identified in *Croton bonplandianus*in Maharashtra, India (Naik *et al.,* 2023). Characterization of phytoplasmas was described on 16S ribosomal gene sequence and 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 transmits the Ziziphus 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, strain virulence, different 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.

Declaration of competing interest

We declare no conflict of interest by the authors.

Data Availability Statement

All data are incorporated into the article and its online supplementary material.

Credit authorship contribution statement

Murali R: survey, conceived the idea, microscopy, investigation, and drafted the original manuscript. **Shambhu Kumar:** Funding acquisition, and Project administration, Resources, Supervision, Validation, review & editing. **Jain Marry Jose**: performed DNA extraction PCR and assisted in molecular analysis. **Mahadevakumar S:** molecular phylogeny analysis and interpretation, Validation of data, review & editing. **Jithu U Krishnan:** Validation of

data, review & editing. **Donald James**: Validation of data, review & editing. All the authors have read the manuscript and agreed for publication.

Ethical approval

This article contains no studies with human participants or animals performed by any of the authors.

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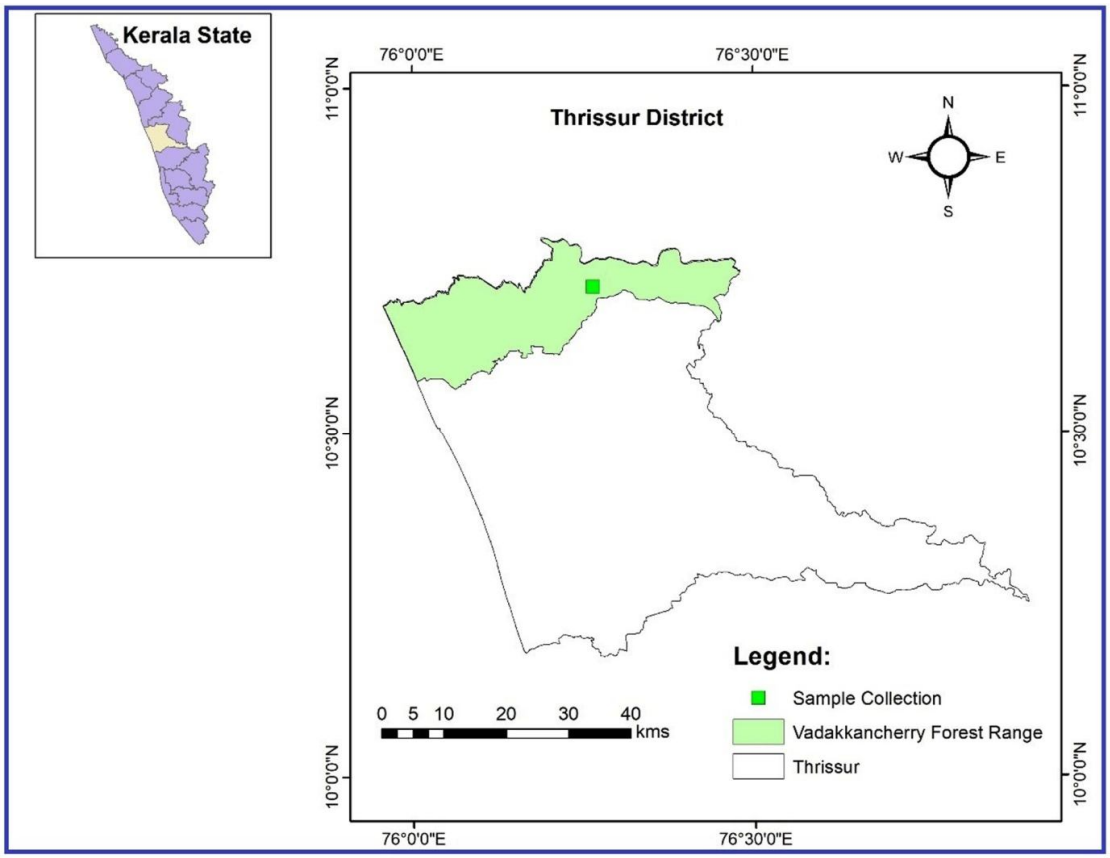

Figure 1: The study area map

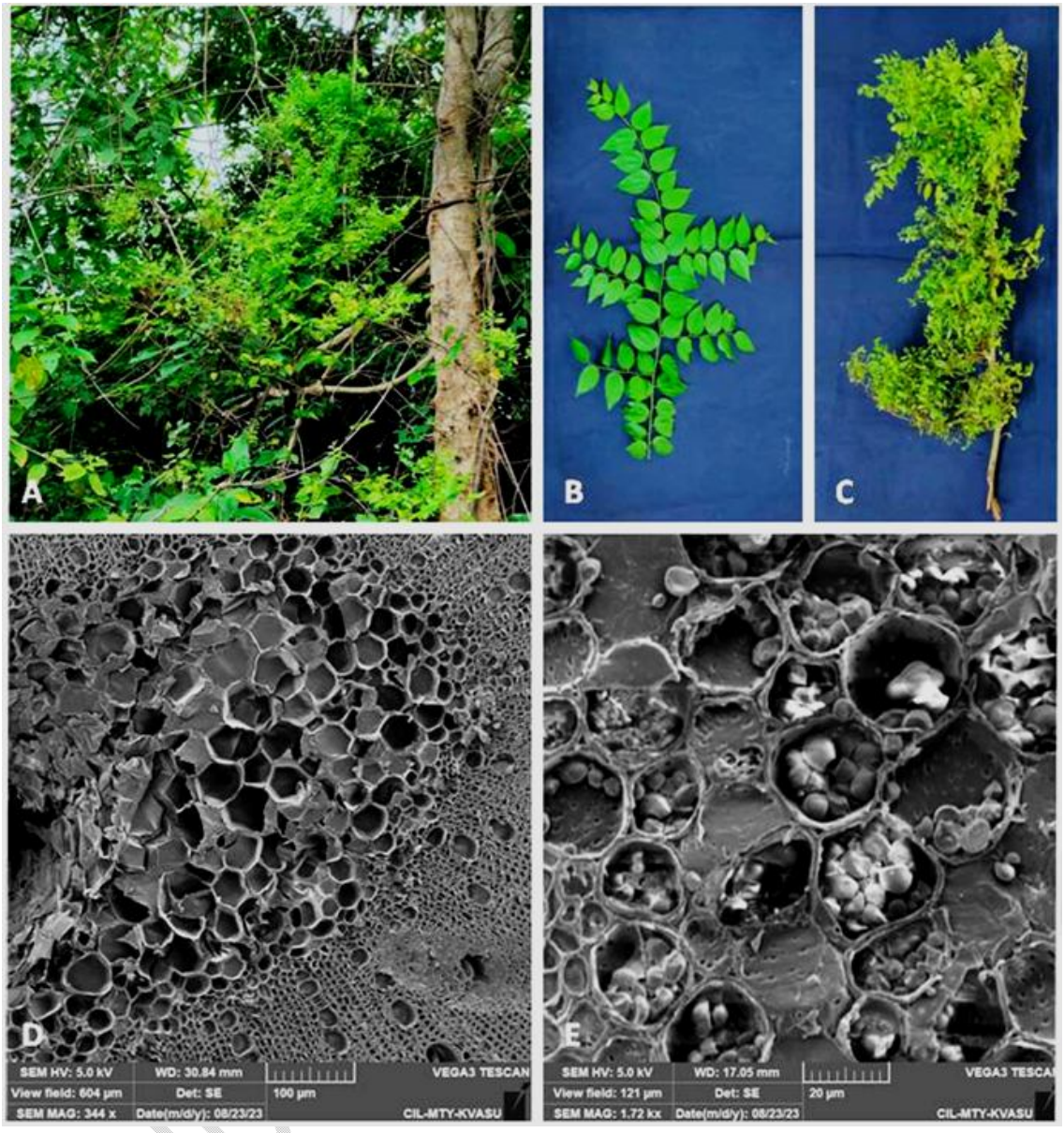

Figure 2: Symptoms on *Ziziphus oenoplea*in wild **A.** Severe incidence of little leaf and witches' broom disease on *Ziziphus oenoplea***B**. Healthy twig with leaves **C**. Little leaf and witches' broom symptoms **D**. SEM image of Phloem cells of healthy *Ziziphus* plant **E**. SEM image of Phloem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

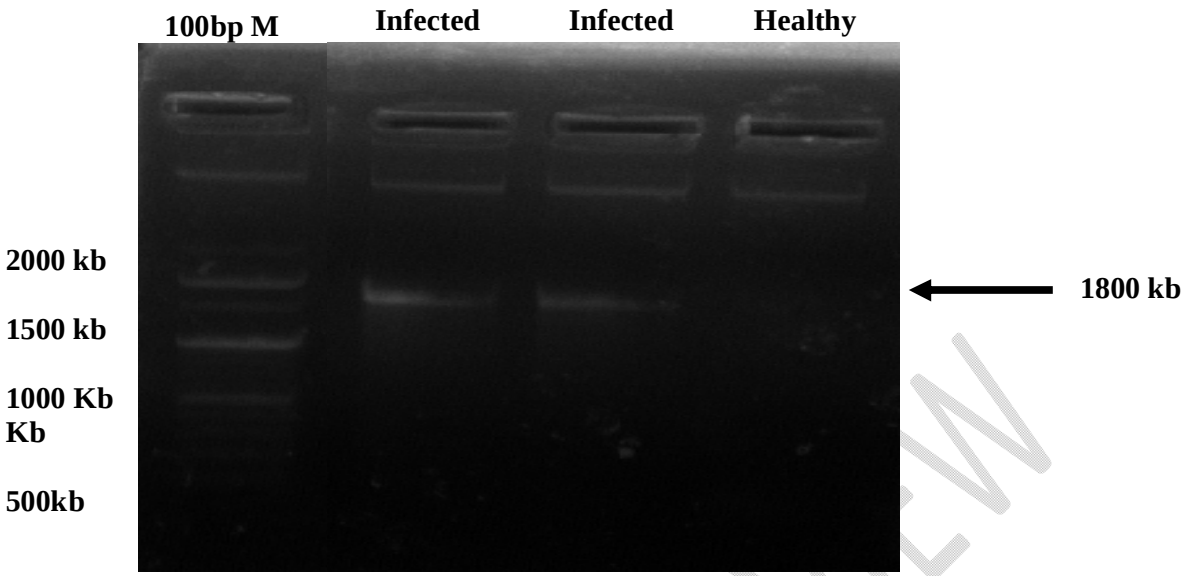

Figure 3: Detection of phytoplasma in little leaf and witches broom infected Ziziphus plant samples through direct PCR using P1and P7 primers

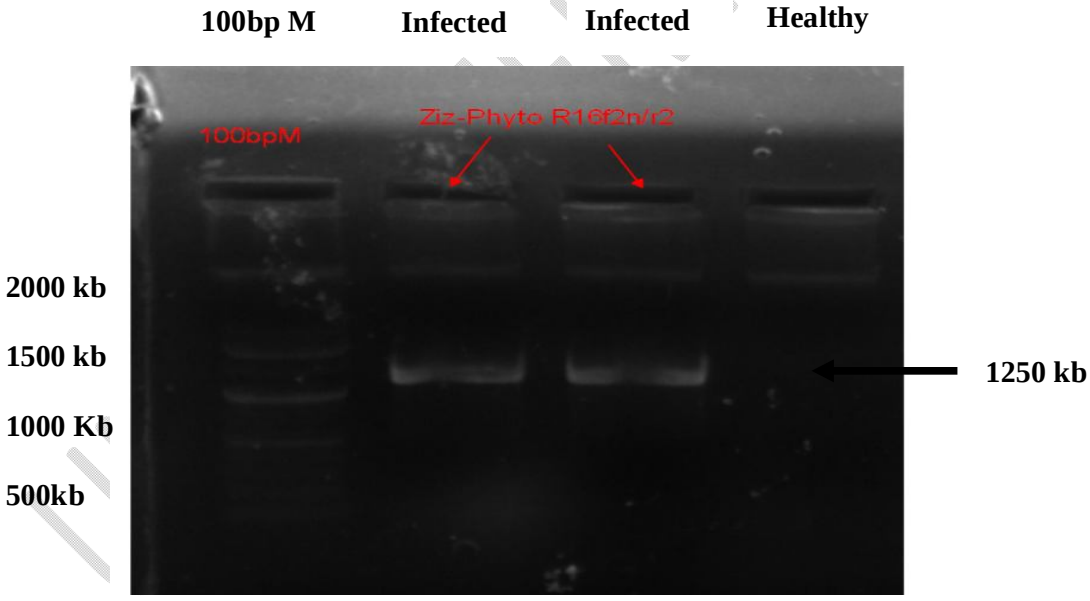

Figure 4: Detection of phytoplasma in little leaf and witches broom infected Ziziphus plant samples through nested PCR using R16F2n and R16R2 primers.

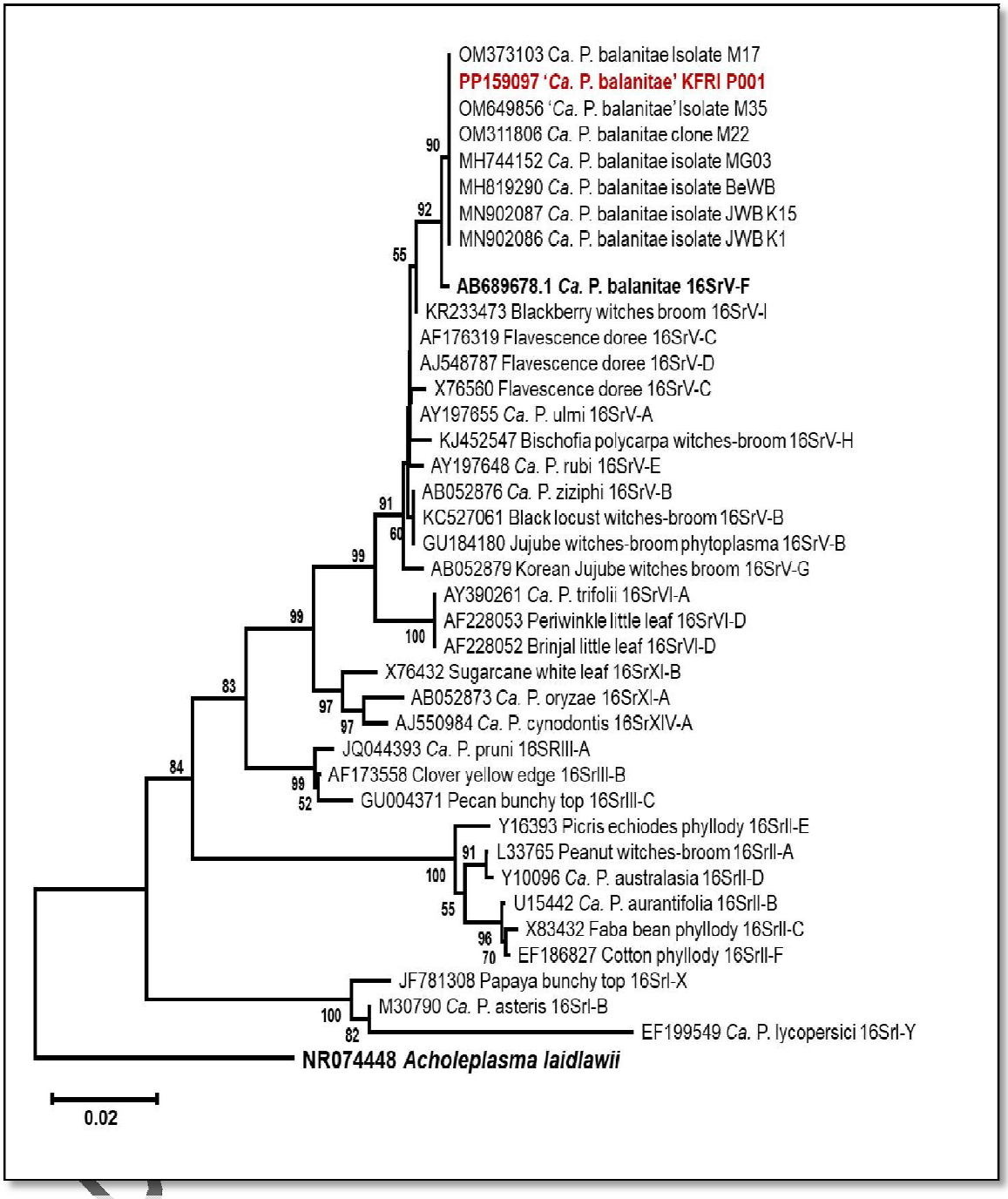

Figure 5: Phylogenetic tree generated from Maximum–Likelihood method (ML) with selected phytoplasma 16S rRNA gene sequences. The sequence of the phytoplasma from this study is marked in red.