J. Phytopathology **156**, 758–761 (2008)
© 2008 The Authors
Journal compilation © 2008 Blackwell Verlag, Berlin
doi: 10.1111/j.1439-0434.2008.01442.x

Short Communication

Division of Crop Protection, Indian Institute of Spices Research, Marikunnu, Calicut, Kerala, India

Occurrence and Partial Characterization of a Phytoplasma Associated with Phyllody Disease of Fennel (*Foeniculum vulgare* Mill.) in India

Alangar I. Bhat[1], Mary V. Jiby[1], Muthuswamy Anandaraj[1], Vemulapati Bhadramurthy[1], Kirtikumar D. Patel[2], Nareshkumar R. Patel[2], Rakeshkumar K. Jaiman[2] and Abbasbhai V. Agalodia[2]

Authors' addresses: [1]Division of Crop Protection, Indian Institute of Spices Research, Marikunnu, Calicut 673 012, Kerala, India; [2]Main Spices Research Station, Sardhar Krushinagar Dandiwada Agricultural University, Jagudan 382710, Gujarat, India (correspondence to A. I. Bhat. E-mail: aib65@yahoo.co.in)

Received August 28, 2007; accepted March 24, 2008

Keywords: fennel, phyllody disease, peanut witches broom phytoplasma, '*Candidatus* Phytoplasma aurantifolia', 16S rDNA sequence

Abstract

In India, fennel showing symptoms of phyllody disease were noticed since 2003. As symptoms were suggestive of a phytoplasma infection, tissues were assayed by nested PCR using universal primers that amplify a 1.2-kb phytoplasma 16S rDNA fragment. The phytoplasma specific fragment was amplified from diseased plants, but not from healthy plants, indicating that a phytoplasma was associated with fennel phyllody (FP) disease. On the basis of 16S rDNA sequence identity and phylogenetic relationship, it is concluded that FP belongs to peanut witches' broom group and may be considered as a strain of the '*Candidatus* Phytoplasma aurantifolia'. This is the first report of occurrence and association of phytoplasma in fennel.

Introduction

Phytoplasmas are wall-less non-helical mollicutes causing diseases characterized by flower malformation, growth aberrations, yellowing and decline in many plant species (Lee et al., 2000). Based on phylogenetic analysis of 16S rDNA sequences and ⁄or RFLP analysis of PCR amplified 16S rDNA, 15 distinct phytoplasma groups were identified (Lee et al., 2000; Bertaccini, 2007). The Phytoplasma Working Team of the International Research Project for Comparative Mycoplasmology (IRPCM) adopted the '*Candidatus* Phytoplasma' genus for formal classification of phytoplasmas and based on diversity within '*Ca*. Phytoplasma' several species level taxa were identified (IRPCM Phytoplasma⁄Spiroplasma Working Team-Phytoplasma taxonomy group, 2004).

Fennel (*Foeniculum vulgare* Mill.) is one of the important seed spices cultivated throughout temperate and subtropical regions of the world including India for its aromatic fruits used as culinary spice. For the first time during the year 2003, a phyllody type of disease was noticed in the fennel crop grown in Mehsana area of Gujarat, India. The incidence of the disease ranged from 1 to 7% and was characterized by malformation of normal flower to a completely vegetative branch and phyllody symptoms. This paper reports symptomatology, detection and sequencing of 1.2 kb fragment of 16S rDNA of the phytoplasma from phyllody affected fennel.

Materials and Methods

Ten phyllody affected fennel samples collected from Mehsana district of Gujarat, India were used in this study. Inflorescences collected from asymptomatic fennel plants were used as healthy control. Total DNAs were extracted from healthy and malformed fennel using DNeasy Plant mini kit (Qiagen, Chatsworth, CA, USA). Total DNAs extracted from a known phytoplasma (periwinkle little leaf) was used as positive control.

The total genomic DNA was subjected to nested PCR using universal primers to amplify a specific sequence within the 16S rDNA of phytoplasmas (Gundersen and Lee, 1996). Primers P6 (5′ CGGTAGGGATCACTTGTTACGACTTA 3′) (Deng and Hiruki, 1991) and SN910601 (5′ CGAAAAAACCTTACCAGGTCTTTG 3′) (Namba et al., 1993) were used for first round amplification of the 16S rDNAs. For the second round, to amplify an internal fragment of the 16S rDNA, primers R16F2n (5′ GAAACGACTGCTAAGACTGG 3′) and R16R2 (5′ TGACGGGCGGTGTGTACAAACCCCG 3′) (Lee et al., 1993) were used. The PCR reaction contained 50 ng each of the primers, 1.5 U *Taq* Polymerase, 1× PCR buffer, 2.5 mm $MgCl_2$ and 10 μm each of the dNTPs, 5μl template DNA and 31.5μl of sterile water. Amplification

was performed in a thermal cycler programmed for one cycle of 94°C for 3 min followed by 35 cycle reaction profile involving 30 s of denaturation at 94°C, 60 s of annealing at 53°C and 90 s of extension at 72°C and single cycle of final extension at 72°C for 10 min for the first round amplification. The reaction product (1 μl) of first round amplification was used as template for the second round of amplification with similar reaction profile except for the annealing step which was carried out at 56°C.

Following PCR, reaction products were analysed by agarose gel electrophoresis, expected band excised from the gel and purified using Perfect prep gel clean up kit (Eppendorf, Hamburg, Germany). The PCR product was then ligated into pTZ57R/T vector (Fermentas, Hanover, MD, USA) and competent *Escherichia coli* (strain JM 109) were transformed by following standard procedures. Recombinant clones were sequenced using automated sequencing of both the strands.

Multiple sequence alignments were made using Clustal W and phylogenetic tree was constructed by Neighborhood Joining method in Clustal X and a rooted tree was generated using TREEVIEW. Bootstrap analysis with 1000 replicates was carried out to check the solidarity of tree. The 16S rDNA nucleotide sequences of other phytoplasma isolates used for comparison were obtained from GenBank.

Results and Discussion

In phyllody affected fennel plants, the entire inflorescence is malformed that gives witches' broom appearance (Fig. 1a). The peduncle is shortened and pedicel is enlarged measuring up to 10 cm (Fig. 1b). The petals become sepaloid and elongated but the inflexed notched apex are retained (Fig. 1c). From individual flowers, further branching occurs and flower like structures are borne on long stalks (Fig. 1d). In some cases, the filament get elongate up to 2 cm instead of normal 2 mm and produce sagitate anthers (Fig 1e). At times, part of the stamen is modified into leaf like structure and a single elongated anther lobe could be seen attached to a leaf-like structure (Fig. 1f).

When the first round PCR products were reamplified in the nested PCR, a DNA fragment of 1.2 kb

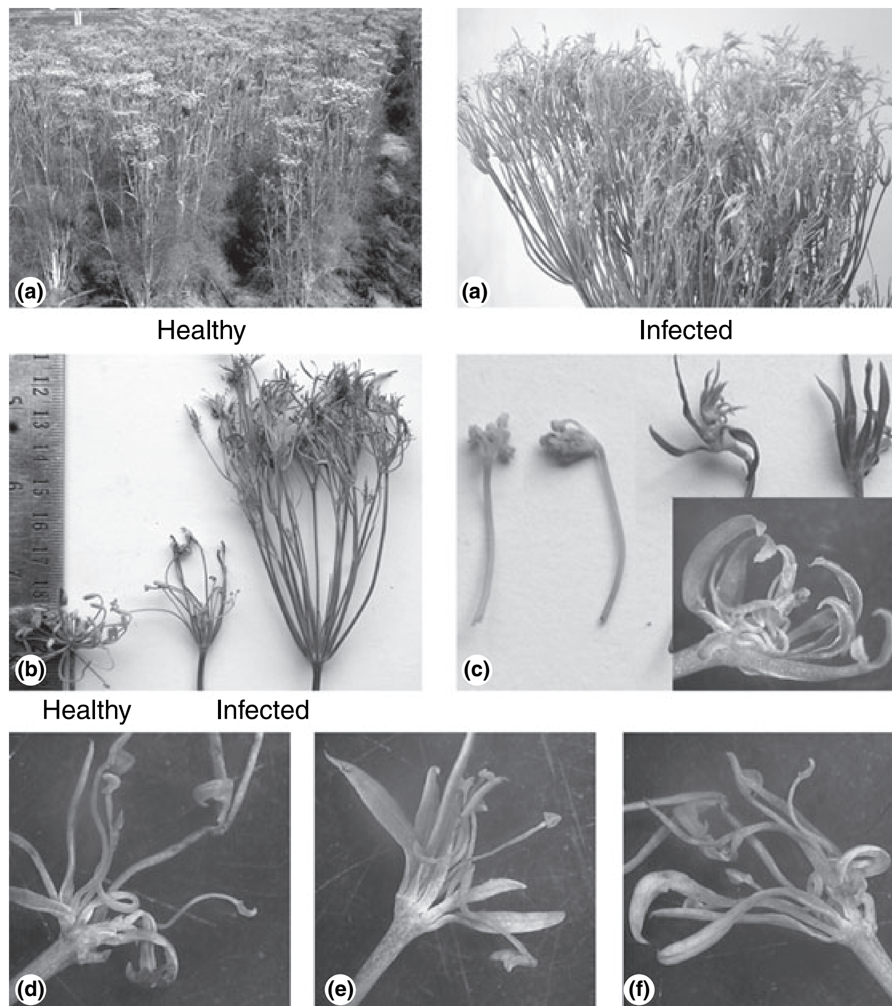

Fig. 1 Symptomatology of phyllody in fennel. (a) Healthy and infected inflorescence, (b) healthy and infected umbel, (c) different stages of affected flower and close-up view of a affected flower (inset), (d) close-up view of proliferation of an affected flower, (e) close-up view of an affected flower showing elongated filament, (f) close-up view of flower showing malformed anther

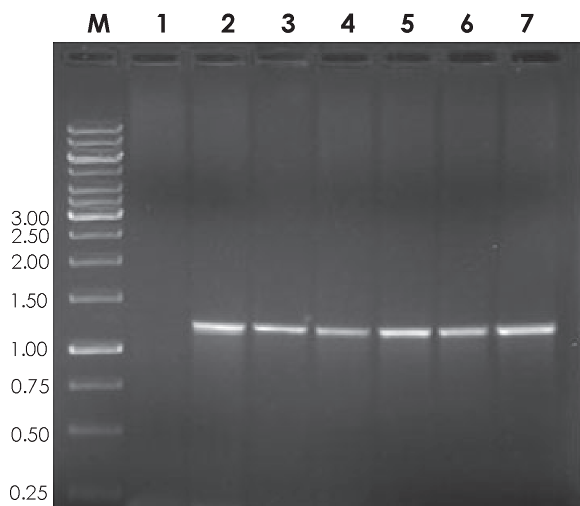

Fig. 2 Agarose gel showing nested- PCR amplification of 1.2 kb fragment of 16S rDNA. Lane M: 1 kb DNA size ladder; lane 1: healthy fennel (negative control); lane 2: periwinkle little leaf (positive control); lanes 3–7: phyllody affected fennel

was obtained in all 10 diseased samples and positive control but not in asymptomatic plants (Fig. 2). This DNA fragment was cloned and sequenced. The sequenced region containing 1239 bases was deposited in GenBank and its accession number is EF584107. The BLAST analysis brought several isolates of phytoplasma belonging to the phylogenetic group, peanut witches' broom (16Sr II) in the '*Ca*. Phytoplasma aurantifolia' as closer to the present fennel phyllody (FP) isolate. Hence, sequence analysis was extended to all IRPCM listed isolates belonging to the 16S rDNA group II (IRPCM Phytoplasma/Spiroplasma Working Team-Phytoplasma taxonomy group, 2004) and one representative isolates each belonging to other 16S rDNA groups. Results of sequence analyses showed that FP shared maximum sequence identity (97.5–99.3%) with phytoplasma isolates belonging to peanut witches' broom group (16Sr II). Within 16S rDNA group II, FP showed maximum identity (99.3%) with phytoplasma associated with papaya mosaic disease

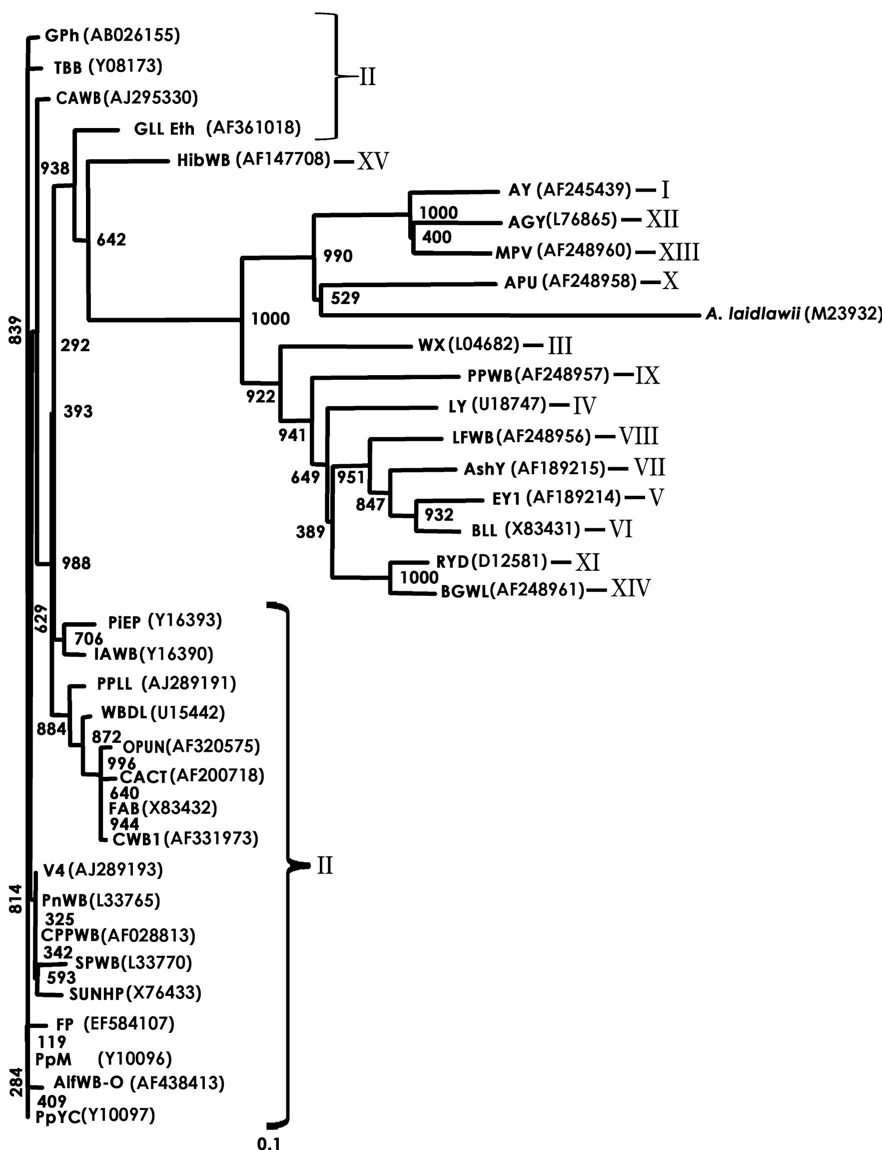

Fig. 3 Phylogram drawn by Neighborhood Joining method in Clustal X (1.81), illustrating phylogenetic relationships based on the multiple alignments of 1.2 kb sequences of 16S rDNA from 34 distinct isolates of phytoplasma representing different groups and fennel isolate (FP). *Acholeplasma laidlawii* was used as outgroup. Sequences for comparisons were obtained from GenBank and accession numbers are shown in parentheses. 16S rDNA group affiliations are given in Roman numerals. Designation given to each of the isolates along with associated plant disease and their corresponding phylogenetic group reported previously (IRPCM Phytoplasma/Spiroplasma Working Team-Phytoplasma taxonomy group, 2004). The bootstrap values are shown at the individual nodes

(PpM) followed by 99.1% identity with phytoplasma associated with papaya yellow crinkle (PpYC) and sweet potato little leaf phytoplasma (V4) and 99% with *Phytoplasma* sp GPh and Chinese pigeon pea witches'-broom (CPPWB). The lowest identity of 97.5% was seen with five different isolates (GLL-Eth, CACT, OPUN, FAB and CWB1). In contrast, identities of FP with 14 other groups of phytoplasma were in the range of 88–95.8%. Phylogenetic tree constructed using these sequences also revealed that FP was most closely related to members of 16S rDNA group II that is well separated from other groups (Fig. 3). Of the other groups, isolate HibWB representing 16S rDNA group XV was found closer to FP.

Sequence similarities between two distinct groups of phytoplasma range from 88 to 94% (Lee et al., 1993, 2000). As the sequence similarity of FP with members of peanut witches' broom group was above the threshold level of 94%, it is proposed that fennel phytoplasma should be regarded as a member of peanut witches broom group. So far one '*Ca*. Phytoplasma' species belonging to 16S rDNA II group, '*Ca*. Phytoplasma aurantifolia' has been described (Zreik et al., 1995). As FP showed an identity of >97.5% with '*Ca*. Phytoplasma aurantifolia', it may be considered as the strain of the same (IRPCM Phytoplasma/Spiroplasma Working Team-Phytoplasma taxonomy group, 2004). This is the first report of occurrence, symptomatology and partial characterization of the phytoplasma associated with fennel.

References

Bertaccini A. (2007) Phytoplasmas: diversity, taxonomy, and epidemiology. *Front Biosci* **12**:673–689.

Deng S, Hiruki C. (1991) Amplification of 16S rDNA genes from culturable and non-culturable mollicutes. *J Microbiol Methods* **14**:53–61.

Gundersen DE, Lee IM. (1996) Ultrasensitive detection of phytoplasma by nested PCR assays using two universal primer pairs. *Phytopathol Mediterr* **35**:144–151.

IRPCM Phytoplasma/Spiroplasma Working Team-Phytoplasma taxonomy group. (2004) '*Candidatus* Phytoplasma', a taxon for the wall-less, non-helical prokaryotes that colonize plant phloem and insects. *Int J Syst Evol Microbiol* **54**:1243–1255.

Lee IM, Hammond RW, Davis RE, Gundersen DE. (1993) Universal amplification and analysis of pathogen 16S rDNA for classification and identification of mycoplasma like organisms. *Phytopathology* **83**:834–842.

Lee IM, Davis RE, Gundersen DE. (2000) Phytoplasma: phytopathogenic mollicutes. *Annu Rev Microbiol* **54**:221–255.

Namba S, Kato S, Iwanami S, Oyaizu H, Shiozawa H, Tsuchizaki T. (1993) Detection and differentiation of plant pathogenic mycoplasma-like organisms using polymerase chain reaction. *Phytopathology* **83**:786–791.

Zreik L, Carle P, Bove JM, Garnier M. (1995) Characterization of the mycoplasma like organism associated with witches'-broom disease of lime and proposition of a "*Candidatus* Phytoplasma aurantifolia". *Int J Syst Bacteriol* **45**:449–453.