Eur J Plant Pathol (2011) 130:1–4
DOI 10.1007/s10658-010-9736-4

Molecular identification of a phytoplasma associated with Ajwain (*Trachyspermum ammi*) in India

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Accepted: 13 December 2010 / Published online: 28 December 2010

Abstract A new disease of *Trachyspermum ammi*, commonly known as Ajwain in India, was observed in Lucknow, India. The symptoms included small chlorotic leaves, highly proliferating shoots, witches' broom appearance, shortened internodes and an overall stunted growth. Poor flower heads and fruit setting caused considerable yield losses for farmers. In transmission electron microscopy, pleomorphic bodies were detected in the phloem cells of diseased plants but not in those of healthy plants. The disease etiology was investigated using direct and nested polymerase chain reaction with phytoplasma-specific primers, DNA sequencing, and phylogenetic analysis. Phylogenetic analysis indicated that this phytoplasma clustered in the 16SrVI group. A 1,249 bp sequence (FJ970035) of the 16S rRNA gene from the phytoplasma showed 99% homology with the 16S rRNA gene, (FJ427295) of *Ca.* Phytoplasma trifolii strain A6 belonging to the phytoplasma group VI (reported from Iran). This is the first report of phytoplasma infection affecting Ajwain (*T. ammi*).

Keywords Electron microscopy · Polymerase chain reaction · Proliferation · Sequencing · 16S rRNA

Trachyspermum ammi (Apiaceae), commonly known as Bishop's weed or Ajwain, is a small annual bush with many branched leafy stems, feather-like tender leaves and flower heads (Fig. 1a). Its leaves yield crystals of thymol, which is known to have medicinal value. The herb is native to Egypt and is cultivated in the Mediterranean region and in South-West Asian countries. The seed, and especially its essential oil component thymol (4–6%), have high medicinal values. Methanol extracts of *T. ammi* showed significant in vitro inhibitory effect on hepatitis C virus as well as diaphoretic, digestive, carminative, diuretic and expectorant properties (Hussein et al. 2000; Kaur et al. 2009). Diseases caused by fungi and bacteria on *T. ammi* have been reported earlier (Sattar and Alam 1993). The objective of this study was to investigate the presence and nature of a phytoplasma associated with *T. ammi*, and to determine whether local weed plants or crops showing phytoplasma symptoms could be reservoirs of the same agent.

In September of 2007, we observed an unusual disease that caused dwarfing, small chlorotic downward curling of leaves, proliferation of shoots, shortened internodes and lacking of flower heads/fruits in Ajwain (Fig. 1b). Disease incidence was

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Fig. 1 Field view of healthy plants (**a**) and naturally infected twig of Ajwain (*T. ammi*) showing yellowing, little leaf, curling and stunted growth (**b**)

about 20–30% in experimental plots while roughly it would be around 21% per acre. The symptoms resembled those of earlier reported phytoplasma infections (Samad et al. 2006; Tanaka et al. 2007). Subsequent surveys revealed the presence of this disease over the last 3–4 years, scattered throughout plantations. Routine tests for common pathogens (fungi, viruses, bacteria, and nematodes) at that time did not reveal a causal agent. In fact the symptoms did not resemble those produced by viral or fungal diseases. Hence the presence of a phytoplasma was suspected.

Phytoplasmas are single-celled organisms that are similar to bacteria but lack a rigid cell wall. They are obligate parasites and cannot survive apart from a host. They grow and reproduce in the cytoplasm of host cells, both in insect vectors and in plants. For electron microscopy, leaf tissue from diseased plants of Ajwain was excised and prefixed with 3% glutaraldehyde in 0.1 M phosphate buffer (pH 7.0) containing 0.2 M sucrose for 2 h at 4°C, post fixed in Dalton's osmium solution (4% $K_2Cr_2O_7$, pH 7.35, 3.4% NaCl, and 2% OsO_4 in a 1:1:2 ratio) for 2 h on ice (Dalton 1955), dehydrated through graded concentrations of ethanol, transferred to propylene oxide, and embedded in Spurr's resin. Thin sections (90 nm thick) were cut with an ultra microtome, stained with uranyl acetate and observed with a Philips transmission electron microscope (TEM) at 80 kV. Typical phytoplasma-like bodies, spherical, ovoid or pleomorphic particles ranging in size from 400 to 900 nm were observed in ultra-thin sections of mature and immature phloem sieve tube elements of affected plants. These objects were always found in diseased plants, but not in asymptomatic ones. No other microorganism such as bacteria or virus, or virus-induced structures, was noted.

At present, differentiation and classification of phytoplasmas rely mainly on molecular analysis of conserved genes, in particular 16S rRNA gene (Lee et al. 2000). Since the presence of a phytoplasma was suspected, samples were randomly collected for further studies. The leaf tissue with petiole (ca.1 g) was excised from diseased as well as asymptomatic plants of Ajwain, respectively. Total genomic DNA was extracted from Ajwain plants using the cetyl trimethyl ammonium bromide (CTAB) method (Ahrens and Seemüller 1992) on a small-scale (Khanuja et al. 1999). Polymerase chain reaction (PCR) using the universal phytoplasma primers P1/P6 (Deng and Hiruki 1991) followed by nested primers R16F2n/R2 (Gundersen and Lee 1996) amplified 1.5 kb and 1.2 kb fragments, respectively. Thirty-fold diluted initial PCR product was used as template for nested amplification. No amplicon was generated in the case of symptomless plants. The amplicons were analyzed by electrophoresis in a 1.2% agarose gel and visualized in UV following staining with ethidium bromide. Samples from the weeds—Mexican poppy *(Argemone mexicana)*, black nightshade *(Solanum nigrum)*, congress weed (*Parthenium hysterophorus*), False Daisy (*Eclipta alba*) and choulai (*Amaranthus gracilis*) growing close to the Ajwain crop were also collected and found negative for the phytoplasma test. PCR products amplified from Ajwain plants were ligated into the plasmid vector using the TOPO TA

Cloning® kit (*Invitrogen*, USA). Transformation and selection of recombinant clones was carried out according to the manufacturer's recommended protocol. Recombinant plasmid DNA was isolated, purified and restricted with *Eco*RI. The released inserts were identified by comparing their size with that of the PCR-amplified fragment and subsequently sequenced using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) on an automated capillary DNA sequencer platform (3130XL Genetic Analyzer, Applied Biosystems). The 1.25 kb sequence (1,249 nucleotides) of the 16S rRNA gene of the TLL phytoplasma strain Ta12 was deposited in GenBank (NCBI) under the accession no. FJ970035 and was analyzed using BLAST (NCBI). From these results, we designated this phytoplasma as the Trachyspermum little leaf (TLL) phytoplasma.The nucleotide sequences of the 16S rRNA genes from various sources were aligned for phylogenetic analysis. A phylogenetic tree was constructed using the neighbour-joining method. A detailed comparison of the TLL phytoplasma 16S rRNA sequence revealed its highest (99%) identity to the 16S rRNA gene (FJ427295) of *Ca.* Phytoplasma trifolii strain A6 of the phytoplasma group VI, reported from Iran. The Ajwain sample clustered with high confidence with the clover proliferation/phytoplasma trifolii (16SrVI) group of phytoplasmas, *Ca*. Phytoplasma trifolii strain 6-FJ427295, Iran and Portulaca little leaf - EF651786, India (Fig. 2). This is the first record of 16SrVI group of phytoplasma on *T. ammi* as no phytoplasma had previously been reported on it in India or anywhere in the world.

In India, 16SrVI phytoplasmas (Clover proliferation group) have been detected in association with little leaf of brinjal, (Martini et al. 2007), witches' broom of Ashwagandha (Samad et al. 2006), little leaf of Portulaca (Samad et al. 2008), Datura (Raj et al. 2009) as well as in tree plants (Gupta et al. 2010). Other local plants were also tested earlier and phytoplasma infections on some of them were reported [Portulaca (Ajayakumar et al. 2007), Periwinkle (Samad et al. 2005), Bhumyamalaki (Samad et al. 2004), Psyllium (Samad et al. 2002a) and Fennel (Samad et al. 2002b)]. Our initial analysis indicates that the little leaf disease of Ajwain is a phytoplasma from the Clover proliferation (16Sr VI) group of phytoplasma. This also confirms that 16SrVI phytoplasmas are widespread in India, and hosted by several plant species. The possibility that portulaca or periwinkle may act, as a reservoir of the phytoplasma cannot be ruled out as these are found near the Ajwain crop. Although a number of specific phytoplasma

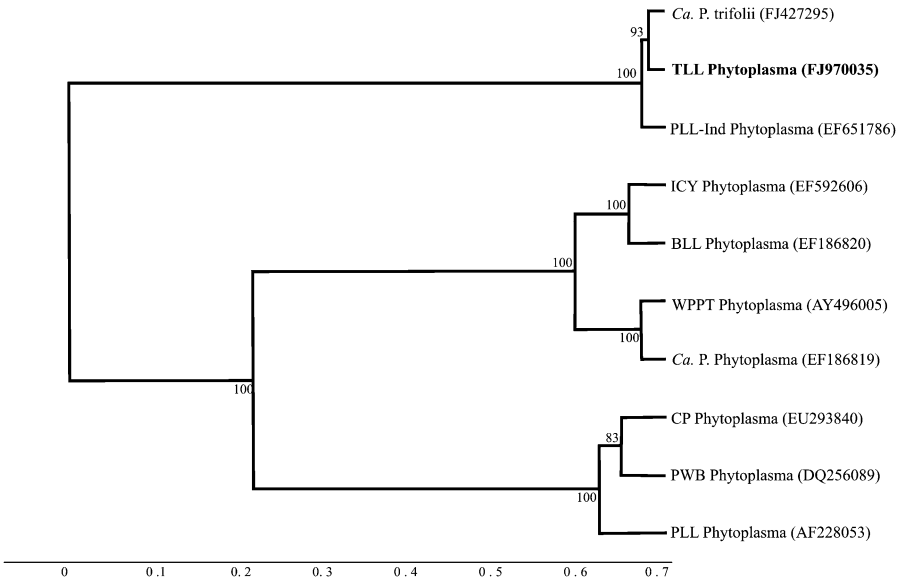

Fig. 2 Phylogenetic relationship analysis, comparing the partial 16S rRNA gene sequence of the TLL phytoplasma with known members of 16SrVI group phytoplasma. GenBank accession numbers for sequences are given in parentheses. (Abbreviations- *Ca.*P. trifolii = *Candidatus* Phytoplasma trifolii; TLL = *Trachyspermum ammi* little leaf; PLL-Ind = Portulaca little leaf India; ICY = Iranian cabbage yellows; BLL = Brinjal little leaf; WPPT = Washington potato purple top; CaP = Catharanthus phyllody; CP = Chinese potato; PWB = Potato witches'-broom; PLL = Periwinkle little leaf)

vectors have been detected in India, nothing is known about the presumed leafhopper that transmits phytoplasmas to Ajwain and other medicinal crops.

Our observations also indicated that severe disease symptoms occurred only on farms producing the same cultivars on the same land, which might be due to better compatibility among host/pathogen and vector. Although assessment of economic loss is not available, a systemic infection is expected to affect adversely standard productivity of Ajwain in the near future. This finding also suggests that these infections may contribute directly or indirectly to premature mortality of this species. Therefore, these organisms may be a critical factor in the etiology of Ajwain degeneration, but further research is needed to verify the exact role of the phytoplasmas.

Acknowledgments We thank Director, CIMAP for providing the necessary facilities and Dr. Ajit K. Shasany, Scientist, CIMAP for scientific help/analysis during the course of investigations. The help of the National Gene Bank for Medicinal and Aromatic Plants at CIMAP, Lucknow and its curator, Dr Anil K Gupta, in providing the Ajwain germplasm is also acknowledged.

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