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Research Article

Alternative weed hosts harbors 16SrII group phytoplasmas associated with little leaf and witches' broom diseases of various crops in India

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Abstract

The disease symptoms characterised by little leaf, phyllody, stunting and branch proliferation were observed during a field survey conducted from June 2015 to January 2016 on common invasive weeds *Cleome viscosa* (tick weed), *Trichodesma zeylanicum* (cattle bush) and *Tephrosia perpurea* (wild indigo), which were found infected with peanut witches' broom phytoplasmas (16SrII group) strains. Phylogenetically similar phytoplasma strains were also detected in symptomatic samples of *Sesamum indicum* (sesame), *Vigna unguiculata* (cow pea), *Phaseolus vulgaris* (French bean), *Dendrocalamus strictus* (bamboo) and *Carica papaya* (papaya) from the same or adjacent fields. This is the first report of 16SrII-D group phytoplasmas ('*Candidatus* Phytoplasma aurantifolia'-related strains) associated with witches' broom disease of *C. viscosa*, *D. strictus* and yellow stunting disease of *C. papaya*. The association of 16SrII-C group phytoplasmas with witches' broom disease of *T. zeylanicum* and *T. purpurea* is also reported for the first time. From the obtained results, it can be inferred that the weed hosts harbors 16SrII group phytoplasma strains associated with little leaf and witches' broom diseases of economically important grain legume crop species like *S. indicum*, *V. unguiculata* and *P. vulgaris*.

Keywords: 16SrII phytoplasmas, *Cleome viscosa*, *Trichodesma zeylanicum*, *Carica papaya*, *Dendrocalamus strictus*, *Sesamum indicum*, *Vigna unguiculata*, *Phaseolus vulgaris*

Introduction

The tick weed (*Cleome viscosa* L.) is an annual herbaceous weed which grows throughout the Indian subcontinent; also valued for its seeds used as a condiment in northern parts of India. Though it is not grown as crop, its leaves have numerous medicinal properties which include being anti-inflammatory, antidiarrheal, antimalarial, rubefacient and vesicant, and are also used as an external application to skin infections, uterine diseases and ulcers (Maikhur *et al.*, 2000). The cattle bush [*Trichodesma zeylanicum* (rm. f.) R. Br.] is a small, commonly found, herbaceous weed that also has medicinal and nutritional properties (Ngonda, 2014). *Tephrosia purpurea* L. Pers. (Fabaceae) is another common Indian weed which was already reported infected with 16SrII group phytoplasmas (Yadav *et al.*, 2014). In the current study symptomatic samples of these weed plants were collected from the fields of economically important plants like *Carica papaya* L. (papaya), *Dendrocalamus strictus* (Roxb.) Nees. (bamboo) and grain legume crops like *Sesamum indicum* L. (sesame), *Vigna unguiculata* L. (cowpea) and *Phaseolus vulgaris* L. (French bean) and tested to verify the presence of phytoplasmas.

The production of papaya saw increasing trend from 1.58 to 5.54 million tons with the average production 3.04 million tons in last 15 years, making India the largest producer of papaya on earth. Bamboo too is economically important plant in India and plays significant role socio-economic status of people engaged

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in its cultivation (Yadav *et al.*, 2015). India also is largest producer of pulses with 14.64 million tons of average production recoded since last 15 years (1998 to 2013). The legume grains are important ingredient of Indian diet and plays important role in Indian economy.

'*Candidatus* Phytoplasma' (Phylum, Tenericutes; Class, Mollicutes) is an endophytic, pathogenic bacterium known to infect many economically important plants and crop species (Dickinson *et al.*, 2013). The typical symptoms shown by phytoplasma infected plants include yellowing or reddening of the leaves, shortening of the internodes leading to stunted growth, smaller leaves and excessive proliferation of shoots resulting in a broom phenotype, loss of apical dominance and phyllody (Lee *at al.*, 2000). Continuous efforts are being made to detect the phytoplasma in plants, alternative hosts and insect vectors to understand the phytoplasma epidemiology.

Materials and Methods

Symptomatic plant samples of tick weed and cattle bush were collected independently from the fields of sesame, cowpea and French bean from Akola and Solapur regions, while wild indigo plant samples were collected from fields of Pune, in and around papaya orchards. At the same time symptomatic samples of papaya, sesame, cowpea and French bean were also collected. Symptomatic bamboo samples were collected from the countryside of Pune and Aurangabad districts, Maharashtra. Asymptomatic samples from each plant species were also collected separately. The disease incidence was calculated by counting symptomatic plant samples versus total number of asymptomatic plants in the given field (papaya and bamboo) or in given quadrant (grain legume crops).

To confirm the presence of phytoplasmas, 25 mg of leaf tissue from representative symptomatic and asymptomatic plants were used for total DNA extraction using MO BIO PowerPlant® Pro DNA Isolation Kit. The genomic DNA from 4 tick weed, 2 cattle bush, 8 sesame, 3 cowpea, 3 French bean, 3 papaya, 3 bamboo and 2 wild indigo plants was extracted. The phytoplasma 16S rRNA gene was amplified from 50 ng of DNA samples using primers P1 (Deng and Hiruki, 1991) and P7 (Schneider *et al*, 1995) and in case of bamboo, cowpea, papaya and cattle bush samples, it was followed by nested PCR with primers R16F2n and R16R2 (Gundersen and Lee, 1996).

PCR products were purified and sequenced directly using bacterial universal primers 343R, 704F, 907R, 1028F and 1492R (Baker *et al.*, 2003). The obtained 16S rRNA sequences were analysed using EzTaxon database (Kim *et al.*, 2012) to find the closest match, deposited in the GenBank database and used to verify their phylogenetic relationships with those of other phytoplasmas using MEGA 6 (Tamura *et al.*, 2013).

Computer simulated RFLP analysis of the 16S rRNA gene was performed on obtained sequences using iPhyClassifier (Zhao *et al.*, 2009). Each sequence was digested *in silico* with 17 restriction enzymes and separated on 3% virtual gels as recommended by Wei *et al.* (2007). Profiles obtained for the phytoplasmas associated with tested plants were compared with published RFLP patterns (Lee *et al.*, 1998) of phytoplasma representatives of other described ribosomal groups.

Results and Discussion

Field observations revealed the presence of typical witches' broom and little leaf symptoms in tick weed, cattle weed, wild indigo and bamboo samples. Proliferation of branches, reduction in leaf size, phyllody, splitting of green pods and plant stunting were observed in sesame, cowpea and French bean samples. Proliferation of axillary shoots, interveinal chlorosis, little leaf and leaf yellowing was observed in papaya samples. The disease incidence observed reached the 2% in cowpea, the 4% in sesame and the 1% in case of French beans. The disease incidence in papaya was from 0.11 to 0.25% in Pune region while the symptoms observed in tick weed and cattle weed are reported here for the first time as a rare event. Also the disease incidence in bamboo too was low to bee calculated considering the scatterd distribution of plantations in the sampled area.

All samples from symptomatic plant showed amplification for phytoplasma 16S rRNA gene plus spacer region and beginning of 23S gene (P1/P7 amplicons), while no amplification was observed in asymptomatic plants. The obtained 16S rRNA gene sequence of sesame, cowpea, French bean, papaya and bamboo (GenBank accession numbers, LT558767, LT558768, LT558769, LT558787and LT558783 respectively) showed 98.31, 98.41, 98.54, 99.39 and 98.46% identity with a strain belonging to the peanut witches' broom group phytoplasma, '*Candidatus* Phytoplasma aurantifolia' (GenBank accession number U15442) when compared using the EzTaxon 16S rRNA database (Kim *et al.*, 2012). Similarly, the obtained 16S rRNA gene sequences obtained from the weed species viz. tick weed, cattle weed and wild indigo, samples (GenBank accession numbers, LT558784 and LT558789

respectively) showed 98.41, 98.11, 98.58, 98.09% identity with the same strain. The virtual RFLP (Zhao *et al.*, 2009) patterns derived from the 16S rRNA sequence of *Tephrosia purpurea* (GenBank accession number LN878981), *Trichodesma zeylanicum* (GenBank accession number LT558789) and *Phaseolus vulgaris* (GenBank accession number LT558769) classified these phytoplasmas in 16SrII-C subgroup (GenBank accession number of reference strain AJ293216); while phytoplasmas from *Carica papaya* (GenBank accession number LT558787), *Cleome viscosa* (GenBank accession number LT558784), *Dendrocalamus strictus* (GenBank accession number LT558783), *Sesamum indicum* (GenBank accession number LT558767) and *Vigna unguiculata* (GenBank accession number LT558768) were classified in the 16SrII-D subgroup (GenBank accession number of reference strain Y10097). The phylogenetic analysis of obtained 16S rRNA gene sequences from symptomatic plant samples confirms that they cluster with phytoplasma strains belonging to 16SrII-C and -D subgroups associated with various crops and weed species reported earlier from India (Figure 1). *C. viscosa* and *T. zeylanicum* represent new plant species hosts for '*Ca.* P. aurantifolia'-related strains, while *D. strictus* and *C. papaya* were confirmed hosts of phytoplasmas belonging to subgroups different from those resported.

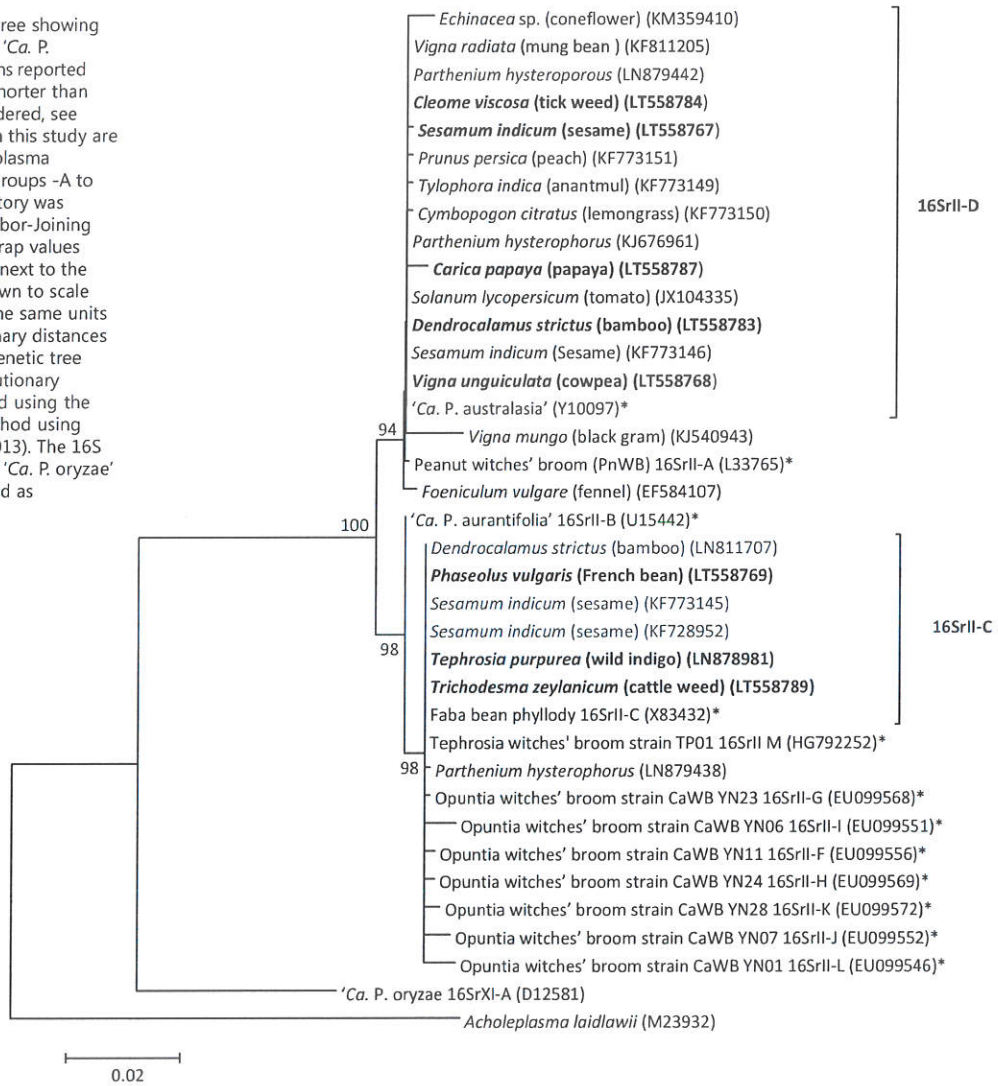

Figure 1. Phylogenetic tree showing the relationships among '*Ca.* P. aurantifolia'-related strains reported from India (sequences shorter than 1,245 bp were not considered, see Table 1). Sequences from this study are shown in bold. *, phytoplasma reference strains of subgroups -A to -M. The evolutionary history was inferred using the Neighbor-Joining method, with the bootstrap values (1,000 replicates) shown next to the branches. The tree is drawn to scale with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree using MEGA 6. The evolutionary distances were computed using the Kimura 2-parameter method using MEGA6 (Tamura *et al.*, 2013). The 16S rRNA gene sequences of '*Ca.* P. oryzae' and *A. laidlawii* were used as

Table 1. List of plant species showing closest match with '*Ca.* P. aurantifolia' reported from India; fetched from GenBank 16S rRNA sequence database and published studies

Symptom	Plant host (common name)	GenBank accession number	Sequence length (bp)	16Sr* group/ subgroup	Geographic distribution	Reference
			Family Acanthaceae			
Witches' broom	*Andrographis paniculata* (kalmegh)	KM359410	1,249	16SrII-D	Lucknow, Uttar Pradesh	Saeed *et al.*, 2015
Yellowing	*Barleria prionitis* (vajradanti)	JF958127	884	ND	New Delhi, Delhi	Kumar *et al.*, 2012
			Family Amaranthaceae			
Phyllody	*Amaranthus* sp. (amaranthus)	EU362627	881	ND	Pantnagar, Uttarakhand	Arocha *et al.*, 2008
Little leaf	*Beta vulgaris* (sugar beet)	GQ869544	785	ND	Bhavanisagar, Tamilnadu	Ramjegathesh *et al.*, 2009, unpublished
			Family Apicaceae			
Phyllody	*Foeniculum vulgare* (fennel)	EF584107	1239	16SrII-C	Gujarat	Bhat *et al.*, 2008
			Family Apocynaceae			
Little leaf	*Tylophora indica* (antamul)	KF773149	1245	16SrII-D	Manesar, Delhi	Priya *et al.*, 2014, unpublished
			Family Asteraceae			
Witches' broom	*Calendula officinalis* (marigold)	EU362631	881	ND	Pantnagar, Uttarakhand	Arocha *et al.*, 2009, unpublished
Little leaf	*Chrysanthemum indicum* (chrysanthemum)	JQ268257	1078	ND	New Delhi, Delhi	Kumar *et al.*, 2012
Leaf yellowing	*Lactuca sativa* (lettuce)	EU362630	881	ND	Pantnagar, Uttarakhand	Arocha *et al.*, 2008
Witches' broom	*Parthenium hysteroporous*	LN879437	1336	16SrII-C	Naldurga, Maharashtra	Yadav *et al.*, 2015
Phyllody		LN879438	1792	16SrII-C	Pune, Maharashtra	
		LN879442	1786	16SrII-D	Pune, Maharashtra	
		KC855731	1249	16SrII-D	Mysor, Karnataka	Sahana *et al.*, 2013, unpublished
		KJ676961	1184	ND	Gorakhpur, Uttar Pradesh	Mall *et al.*, 2014, unpublished
			Family Boraginaceae			
Little leaf	*Trichodesma zeylanicum* (cattle bush)	LT558789	1274	16SrII-C	Solapur, Maharashtra	This study
			Family Brassicaceae			
Proliferation	*Brassica oleracea* (cauliflower)	HQ625432	833	ND	New Delhi, Delhi	Kumar *et al.*, 2011, unpublished
			Family Capparidaceae			
Little leaf	*Cleome viscosa* (tick weed)	LT558784	1792	16SrII-D	Naldurg, Solapur	This study
			Family Caricaceae			
Yellowing, Dieback	*Carica papaya* (papaya)	LT558787	1332	16SrII-D	Pune, Maharashtra	This study
		JQ346525	788	ND	Pune, Maharashtra	Verma *et al.*, 2012
			Family Fabaceae			
Big budLittle leaf	*Phaseolus vulgaris* (French bean)	EU362629	881	ND	Pantnagar, Uttarakhand	Arocha *et al.*, 2008
		LT558769	1754	16SrII-C	Akola, Maharashtra	This study
Witches' broom	*Tephrosia purpurea* (wild indigo)	HG792252	1246	16SII-M	Naldurga, Maharashtra	Yadav *et al.*, 2014
		LN878981	1800	16SrII-C	Pune, Maharashtra	This study
Phyllody	*Vigna mungo* (black gram)	KJ540943	1584	16SrII-D	Tirupati, Andhra Pradesh	Reddy *et al.*, 2014
Witches' broom	*Vigna radiata* (mung bean)	KF811205	1238	16SrII-C	Tirupati, Andhra Pradesh	Saeed *et al.*, 2015
Witches' broom	*Vigna unguiculata* (Cow Pea)	LT558768	1248	16SrII-D	Akola, Maharashtra	This study
			Family Nyctaginaceae			
Little leaf	*Mirabilis jalapa* (beauty of night)	JQ268255	883	ND	New Delhi, Delhi	Kumar *et al.*, 2012
			Family Pedaliaceae			
Phyllody, Witches' broom	*Sesamum indicum* (Sesame)	LT558767	1380	16SrII-D	Akola, Maharashtra	This study
		KF728952	1264	16SrII-D	Kushinagar, Uttar Pradesh	Sajad *et al.*, 2015
		KF773146	1249	16SrII-D	Coimbatore, Tamilnadu	Priya *et al.*, 2014, unpublished
		KF773145	1247	16SrII-D	Raipur, Chhattisgarh	Priya *et al.*, 2014, unpublished
		JX436191 to JX436205; JX448398 to JX448401	<1200	ND	Banawara, Kenkere, Hirebenkal, Raichur, Karnataka; Mawana, Khurja, Badaun, Uttar Pradesh; New Delhi; Walandi, Chakur, Udigir, Ausa, Nilanga, Maharashtra; Mehboobnagar, Telangana, Manjampalli, Andhra Pradesh	Kumar *et al.*, 2012, unpublished

Table 1 continuation

Symptom	Plant host (common name)	GenBank accession number	Sequence length (bp)	16Sr group/ subgroup	Geographic distribution	Reference
			Family Poaceae			
White leaf	*Cymbopogon citratus* (lemongrass)	KF773150	1246	16SrII-D	Manesar, Delhi	Priya *et al.*, 2014, unpublished
Witches' broom	*Dendrocalamus strictus* (bamboo)	LN811707 LT558783	1337 1237	16SrII-C 16SrII-D	Pune, Maharashtra Aurangabad, Maharashtra	This study Yadav *et al.*, 2015
			Family Rosaceae			
Leaf yellowing	*Prunus persica* (peach)	KF773151	1245	16SrII-D	Manesar, Delhi	Priya *et al.*, 2014, unpublished
			Family Solanaceae			
Big bud, Witches' broom	*Solanum lycopersicum* (tomato)	KF975588 JX104335	1880 1698	16SrII-D 16SrII-D	Bangalore Rural, Karnataka Meerut, Uttar Pradesh	Swarnalatha *et al.*, 2014, unpublished Singh *et al.*, 2012

*The group and subgroup level delineation was determined based on sequence size (above 1,245 bp; ND = Not determined.

The finding of 16SrII phytoplasmas in weeds are of epidemiologic relevance since phytoplasmas of same group and subgroups were found associated with bamboo, papaya and grain legume crops during this and previous studies (Table 1). *C. viscosa* and *T. zeylanicum* pose a potential threat as alternate phytoplasma host which can harbor phytoplasmas when crops are off-field. With reference to previous studies (Table 1), 24 plant species, belonging to 13 families have been showing diseases associated with the presence of 16SrII group phytoplasmas. This list includes families Apiaceae, Asteraceae, Brassicaceae, Caricaceae, Fabaceae, Pedaliaceae, Poaceae and Solanaceae enclosing economically important crop, medicinal and aromatic plant species. Since the list also contains plant species know as invasive, noxious weeds like *Parthenium*, *Amaranthus*, tick weed, wild indigo, cattle bush which, this strongly suggest that these weed species act as alternative hosts and need to be studied with equal attention given to crops or other economically important plant species.

The close genetic association of phytoplasma strains found in current and previous studies suggests the presence of common insect vector(s). Weeds are known harboring rich insect fauna including leafhoppers, psyllids and planthoppers which transmit phytoplasmas (Anderson *et al.*, 2004). For example, *Orosius albicinctus* (Cicadellidae) has been reported as a vector of alfalfa witches' broom (Salehi *et al.*, 1995), cucumber phyllody (Azadvar *et al.*, 2005), garden beet witches' broom (Mirzaie *et al.*, 2007), sesame phyllody (Esmailzadeh-Hosseini *et al.*, 2007) and parthenium witches' broom (Yadav *et al.*, 2015) which suggests its possible role in transmitting 16SrII group phytoplasmas. Further studies are required to understand the epidemiology of phytoplasma transmission in diseases of crop plants and alternative weed hosts in India.

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