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ORIGINAL PAPER

Molecular characterization of '*Candidatus* Phytoplasma *a*steris' subgroup I-B associated with sesame phyllody disease and identification of its natural vector and weed reservoir in India

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Abstract An incidence of 12 to 35 % sesame phyllody (SP) disease was observed in sesame fields at Kushinagar and Gorakhpur districts in Uttar Pradesh and Indian Agricultural Research Institute, New Delhi, India during July-October 2013. The association of phytoplasma with sesame phyllody plants at all the three locations and a weed, *Sclerocarpus africanus* Jacq (SA) showing little leaf and witches' broom symptoms at Gorakhpur was confirmed by direct and nested PCR amplification of phytoplasma 16S rDNA. Out of four predominant feeding leafhopper species in the symptomatic sesame fields, viz. *Empoasca prima* (Distant), *Exitianus indicus* (Distant), *Hishimonus phycitis* (Distant) (HP) and *Cofana unimaculata* (Signoret), only *H. phycitis* was found to be associated with phytoplasma in nested PCR assays with phytoplasma universal primer pair R16F2n/R16R2. Transmission test and population dynamics study further confirmed that *H. phycitis* was the proven natural and potential vector to transmit the SP phytoplasma from diseased to healthy sesame plants in transmission assays. BLAST analysis of 1.25 kb 16SrDNA partial sequences of nested PCR products obtained from symptomatic SP plants, weed (SA) and the leafhopper (HP) revealed 99 % sequence identities among themselves and 99 % identity with other reported strains of '*Ca*. P. asteris' (16Sr I group). Phylogenetic analysis also suggested the closest phylogenetic relationship of SP, SA and HP phytoplasmas with those of '*Ca*. P. asteris' group. RFLP analysis of R16F2n/R16R2 primed 16S rDNA sequences of SP, SA and HP phytoplasma isolates using *i*PhyClassifier online tool with 17 selected restriction enzymes confirmed the association of '*Candidatus* Phytoplasma asteris' subgroup B with SP, SA and HP in India. The identity of natural vector and new alternative host of SP phytoplasma in the present study suggested that these may act as potential/natural source for secondary spread of sesame phyllody phytoplasma.

Keywords *Hishimonus phycitis* · *Sclerocarpus africanus* · Molecular detection · Sesame phyllody · 16SrI-B subgroup · Phylogeny · Vector transmission

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Introduction

Sesame (*Sesamum indicum* L.), also known as til and gingerly, is an important and ancient oil-yielding crop, domesticated over 3000 years ago. The sesame seed is a rich source of edible oil. Its oil content generally varies from 46 to 52 % along with unsaturated fatty acids and antioxidant lignans (Uzun et al. 2008; Moazzami and Kamal-Eldin 2006). Sesame is an important oilseed crop of India and is grown in an area of 1.9 mha which ranks first in area (46.5 %) under sesame cultivation in the world. The total annual global sesame seeds production was about 3.84 million metric tonnes of which India produces around 0.81 million metric tonnes (26 %) and stands at second place in world only after China (FAO 2013).

Major factors that limit sesame productivity besides its narrow genetic base are extreme susceptibility to biotic and abiotic stresses. Sesame phyllody (SP), an important disease of sesame is associated with phytoplasma presence and transmitted by leaf hoppers (Vasudeva and Sahambi 1955; Abraham

et al. 1977; Asri 1998; Akhtar et al. 2009) and causes losses up to 80 % (Kumar and Mishra 1992; Salehi and Izadpanah 1992). Phytoplasmas are wall-less prokaryotes that colonize plant phloem and insects and are associated with hundred of diseases worldwide. Phytoplasmas are at priority transmitted by phloem-feeding insects in nature (Weintraub and Beanland 2006; Hogenhout et al. 2008). In sesame phyllody disease, the affected plants become stunted and floral parts being modified into a leafy structure bearing no fruits and giving witches' broom appearance. Since then the disease has now been reported all over the Asia, Africa and North America (Al-Sakeiti et al. 2005; Sertkaya et al. 2007; Akhtar et al. 2009; Win et al. 2010; Pathak et al. 2012; Cengiz et al. 2014). The previous findings indicate that SP was caused by different phytoplasma groups and subgroups, and transmitted by different leafhopper vectors (Cengiz et al. 2014). The sesame phyllody phytoplasma belonging to 16SrI group has been successfully transmitted by grafting and dodder (Akhtar et al. 2009). However, no sap and seed transmission was confirmed (Akhtar et al. 2009; Tan 2010). So many leafhopper species viz., *Neoaliturus haematoceps* and *Circulifer haematoceps* from Iran and Turkey (Salehi and Izadpanah 1992; Kersting 1993), *Orosius orientalis* from Iran, Pakistan, Turkey and India (Hosseini et al. 2007; Akhtar et al. 2009; Pathak et al. 2013; Cengiz et al. 2014) and *O. albicinctus* in Iran (Esmailzadeh et al. 2007) were reported as vectors of sesame phyllody phytoplasmas.

Association of phytoplasma has been confirmed with sesame phyllody disease in India on the basis of symptoms and electron microscopy but the molecular approaches are little attempted (Vasudeva and Sahambi 1955; Abraham et al. 1977; Khan et al. 2007; Manjunatha et al. 2012). Transmission of sesame phyllody by leafhoppers has been reported in India but only up to limited extent by transmission assays and serological detection of phytoplasmas in insects and sesame plants (Srinivasulu and Narayanasamy 1995; Pathak et al. 2013). In this study, attempt has been made to investigate the identity and diversity of the phytoplasmas associated with sesame phyllody disease in India and identification of insect vector and alternative host plants for SPP on the basis of molecular approaches.

Materials and methods

Plant samples survey

A survey was made to record the incidence of phytoplasma disease on sesame and weed species in sesame fields at Indian Agricultural Research Institute New Delhi and two districts of Uttar Pradesh viz., Kushinagar and Gorakhpur during August to October 2013. Two symptomatic sesame plants each from all the three different locations and one weed species (*Sclerocarpus africanus*) from Gorakhpur location showing suspected phytoplasma symptoms were collected in plastic bags and stored in deep freezer at −20 °C for further PCR analysis.

Leafhoppers samples survey

The predominant leafhopper (LH) species feeding on sesame crops in respected survey areas were collected at 10 days interval in the month of July-October 2013 at all the three surveyed locations by sweeping net method. Collected insects were stored in plastic vials and were stored at −20 °C in 70 % ethanol for further analysis. The LH species were taxonomically identified by Department of Entomology, Indian Agricultural Research Institute, New Delhi, India and Network Project on Insect Biosystematics, Department of Entomology, Bangalore, India.

DNA extraction and PCR assays

DNA was extracted from leaf tissues of symptomatic and non-symptomatic sesame plants and weed species and the whole body of ten individuals' leafhopper species of each identified major species from all the surveyed locations by CTAB method (Ahrens and Seemüller 1992). The DNA concentrations were measured with Nanodrop spectrophotometer (ND-1000 UV/VIS, USA).

Extracted DNA from sesame, weed and leafhoppers species were used as template in PCR assays by universal primer pair P1/P6 in first round (Deng and Hiruki 1990; Schneider et al. 1995) followed by R16F2n/R16R2 in second round nested PCR assays (Gundersen and Lee 1996) derived from conserved regions of the 16S rRNA gene. PCR reactions were performed in a Mastercycler (Eppendorf Germany) and the cycling protocol used for the first and nested round PCR as described (Rao et al. 2014). The product of first PCR was diluted 1:4 with sterile water and 2 μl of product of the first round of PCR was used as template in nested PCR using primer pairs R16F2n/R16R2. The DNA isolated from periwinkle infected with toria phyllody phytoplasma (group 16SrIX, pigeon pea witches' broom phytoplasma; Azadvar et al. 2009) and maintained in a greenhouse was used as positive control. The DNA extracted from non-symptomatic sesame, weeds and leafhoppers collected from SP disease free areas was used as negative controls. Twenty-five microlitres of each PCR product was subjected to electrophoresis in a 1.0 % (*w*/*v*) agarose gel, stained with ethidium bromide and observed under UV transilluminator. Nested PCR product (1.25 kb amplicon) was purified using the Wizard^{R} SV Gel and PCR Clean-up System (Promega, USA).

Cloning, sequencing and BLAST analysis

The amplified nested fragments (1.25 kb) of phytoplasmal rRNA gene and spacer region obtained from SP and HP phytoplasma was cloned in TA vector using a cloning kit (RBC TA Cloning Vector Kit) as per manufacturer's instructions. Recombinant plasmids were screened by blue/white colour screening on indicator plates and PCR amplified using the same primer pair. Cloned plasmids were extracted from a selected colony, purified by using (MDI Plasmid isolation kit) and then sequenced in both directions and the 16SrRNA gene sequences for sesame phyllody strains, weed and leafhopper species were submitted to GenBank. Primers for the sequencing PCR product were the same as for the nested PCR amplification. The sequences of PCR products were assembled using DNA baser V.4 program and were further aligned using CLUSTAL W method of Bio-Edit software. Aligned sequences were deposited in NCBI GenBank and used as query sequence in BLASTn search with related submitted sequences in GenBank.

In silico RFLP analysis

The 1.25 kb of 16S rDNA sequences derived from phytoplasmas associated with SP and HP were submitted to iPhyClassifier online tool (http://www.plantpathology.ba.ars.usda.gov/cgibin/resource/iphyclassifier.cgi) and compared with the virtual RFLP gel from 16SrI phytoplasma subgroups by the same restriction enzymes (Zhao et al. 2009).

Phylogenetic analysis

The sequence generated from the present study and twenty two reference phytoplasma strains sequence retrieved from GenBank were used to construct phylogeny by neighbor joining method with 1000 replications for each bootstrap value using MEGA 5.0 software version (Tamura et al. 2011). *Acholeplasma laidlawii* was used as out group to root the tree.

Incidence of SD and leafhoppers dynamics

The yellow sticky traps of 5×10 cm (250 g/m^2) were placed at borders and in between the rows of crop. The traps were hung about the canopy level of the crop. Population dynamics of the identified leafhoppers was calculated by counting the trapped leafhopper species on the yellow sticky insect traps at 10 days interval in sesame fields at Kushinagar and Delhi up to 90 days of collection and was correlated with sesame phyllody incidence in the fields.

Transmission trials

Eggs, nymphs and adults of HP (identified as carrier of phytoplasmas by PCR assays in field collected samples) were collected from the disease free fields and their colonies were established on healthy greenhouse grown sesame plants in pots until the emergence of first generation (approx 3 weeks later). Twenty five individuals LH from established colony were tested by PCR assays for the presence of phytoplasmas, to ensure colonies were phytoplasma free.

For leafhopper transmission assays, seeds of sesame plants were sown in pots and placed in insect free green house inside the cages covered with plastic cylinder of 13″ diameter tighten on top. A total of 20 sesame plants were grown in four pots in cages with 5 healthy sesame plants in each pot. A total of 25 adult HP (from established colonies in greenhouse) after acquisition access feeding of 72 h in SP infected pots were transferred to three of the pots containing 5 healthy sesame plants followed by an inoculation access period of 7 days. One cage with 5 healthy sesame plants was used for control where no HP was released. The HP population in the treated pots was killed using insecticide and plants in each cage were continuously monitored for symptom expression up to 30 days post inoculation. The killed insects and the symptomatic sesame plants in experimental cages after transmission test were collected and further analyzed for the presence of phytoplasma using PCR assays.

Results

Incidence of SP disease & symptomatology on sesame plants and weed species

During survey of sesame fields at Kushinagar and Gorakhpur districts of Uttar Pradesh and Indian Agricultural Research Institute (IARI) New Delhi, India, an incidence of 12–35 % of phytoplasma suspected symptoms like virescence, phyllody and witches' broom was recorded on sesame plants (Fig. 1a–c). Little leaf, witches'broom and chlorosis symptoms was also observed on weed species, *Sclerocarpus africanus* of family asteraceae in sesame phyllody affected fields at Gorakhpur (Fig. 2).

Identification of the leafhoppers specimens collected

Four major leafhopper species feeding on symptomatic sesame plants collected from Kushinagar, Gorakhpur and Delhi sesame fields in the month of July-October 2013, were identified as *Empoasca prima* (Distant), *Cofana unimaculata*,

Fig. 1 Sesame plants phyllody and witches broom (**a**) Kushinagar (**b**) Gorakhpur (**c**) Delhi

Exitianus indicus (Distant) and *Hishimonus phycitis* (HP) (Distant).

Phytoplasma detection by PCR assays

PCR amplification of symptomatic sesame plants from all the three surveyed locations and toria phyllody infected *Catharanthus roseus* positive control yielded approximately ~1.5 kb and ~1.25 kb products in the first round and nested PCR assays, respectively with primer pair P1/P6 and R16F2n/R16R2 (data not shown). However, no amplification was achieved with any of the four leafhopper species and the test weed species analyzed in the first round PCR assays with primer pair P1/P6. In nested PCR analysis, however, ~1.25 kb amplified product was obtained only with leafhopper species, *Hishimonas phycitis* (Distant) from Delhi and Kushinagar locations, and the symptomatic weed species (*Sclerocarpus africanus*) from Gorakhpur (data not shown).

Identification of phytoplasmas through sequence analysis

The 16Sr DNA 1.25 kb sequence data for the SP isolates, *S africanus* and the leafhopper, *H. phycitis* were submitted to GenBank (HP: Delhi isolate Ac No KF826901, UP isolate Ac. No. KC495560; SP: IARI isolate KF744233, UP isolate KF728955, GKP isolate KF744231; SA: GKP isolate KJ561783). BLAST analysis of the 16S rRNA partial gene sequence of phytoplasma isolates of *H. phycitis* (Distant) (Kushinagar and Delhi), *S. africanus* (Gorakhpur) and the sesame phyllody isolates from all the three locations (Kushinagar, Gorakhpur and Delhi) revealed 99 % identity among themselves and with identified isolates of Aster yellow phytoplasma (AY180951), sesame phyllody (KF744231, KF728954), *Stellaria media* phytoplasma (KC623537), *Solanum tuberosum* phytoplasma (KC312703), periwinkle little leaf (EU375834) and bitter gourd little leaf phytoplasma Myanmar (AB741631), all belongs to strains of 16SrI group phytoplasma.

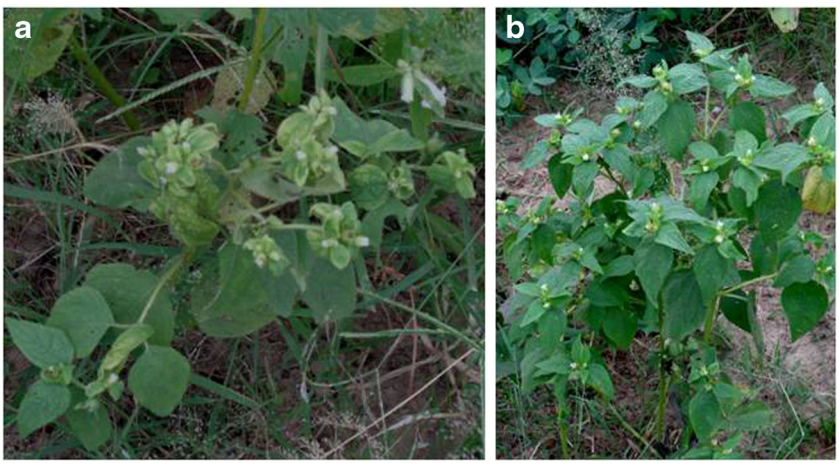

Fig. 2 Little leaf and witches broom symptoms on *Sclerocarpus africanus*

Phylogenetic relationships

All the phytoplasma isolates of SP, HP and SA in the present study clustered together within the phylogenetic tree with members of '*Ca*. P. asteris' group (16SrI) (Fig. 3). Our results confirmed that the phytoplasma isolates associated with the sesame, weed (SA) and the leafhopper (HP) belonged to '*Ca*. P. asteris' group.

RFLP analysis

All the six isolates of phytoplasmas from SP, HP and SA showed 99 % sequence similarity among themselves, therefore we utilized one representative strain each of SP and HP from Delhi for RFLP analayis. Sequence analysis of the SP and HP phytoplasma isolates from Delhi with *iPhy*classifier online tool indicated that the virtual RFLP pattern derived from the 1.25 kb F2nR2 fragment of 16Sr DNA sequence was identical (similarity coefficient 1.00) to the reference RFLP pattern of 16Sr I subgroup B (Ac M30790) (Fig. 4a, b).

Fluctuation of sesame phyllody disease and leafhoppers

The *H. phycitis* was found as the most abundant leafhopper species recorded in sesame fields at Delhi and Kushinagar districts of Uttar Pradesh, India during August to October, 2013 followed by *E. indicus* at Kushinagar and Gorakhpur. However, the green leafhopper, *E. prima* was recorded as most prevalent species at Gorakhpur and *C. unimaculata* at Kushinagar in sesame fields, respectively (Table 1). The high population index of HP in sesame fields at Delhi (255) and Kushinagar (307) were found positively correlated with increasing incidence of SP symptoms in the field (Fig. 5).

Transmission of SDP by Hishimonus phycitis

Since the leafhopper *H. phycitis* was found positive for the phyllody phytoplasma as indicated by the results of PCR and sequence analyses, transmission studies were performed with HP under green house conditions. The leafhopper, *H. phycitis* successfully transmitted the SP phytoplasma from infected sesame plants to healthy sesame plants as indicated by symptom development and nested PCR analysis, which provided evidence for its role as a natural vector under experimental condition employed (Table 1). In the transmission assays, 40–60 % sesame plants in all the three cages inoculated with *H. phycitis* showed typical disease symptoms and were tested positive for the presence of 16SrI group of phytoplasma in PCR analyses (Table 1). Five plants in the control cage with no insect infestations did not display any disease symptoms and were found negative for the phytoplasmas in PCR assays. Sequence analysis of PCR amplicons from experimental

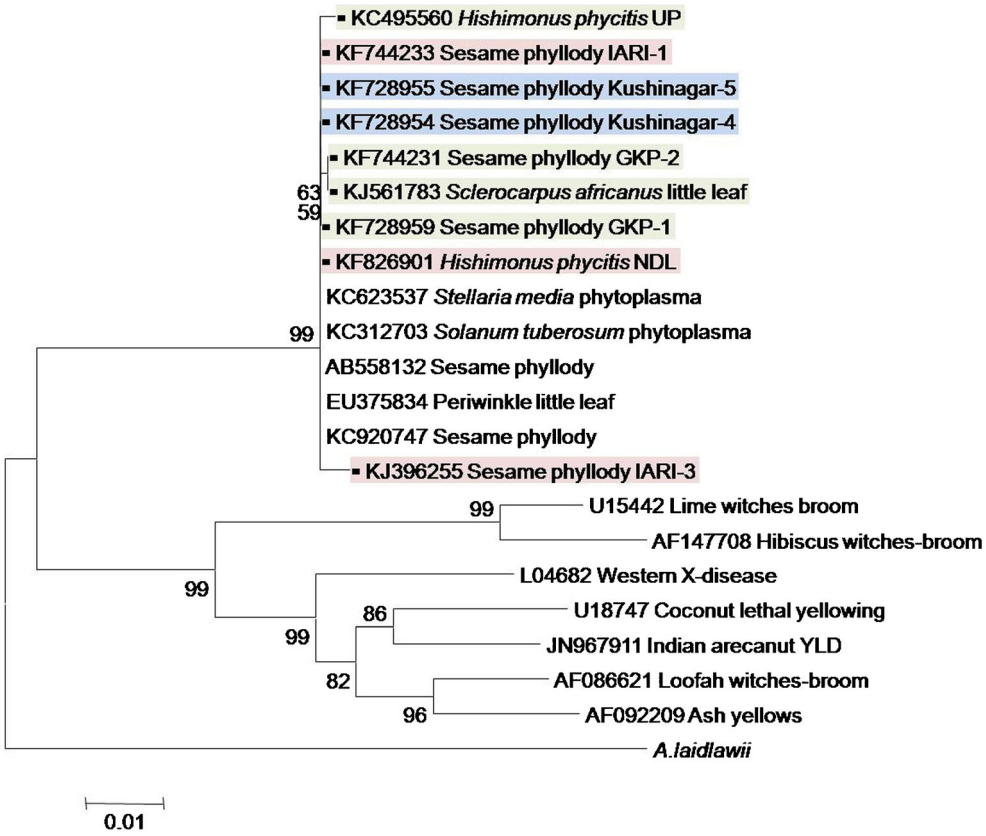

Fig. 3 Phylogenetic tree constructed by neighbor-joining method showing the relationships among sesame phyllody, SA and HP phytoplasma, and reference phytoplasma strains. Accession numbers are specified in the tree. 'Ca. P.' stands for '*Candidatus* Phytoplasma sp.' *A. laidlawii* was used as an out group. Mega 5.0 software was used to construct the tree. Numbers on branches are bootstrap values obtained for 100 replicates (only values above 80 % are shown). The bar represents a phylogenetic distance of 1 %

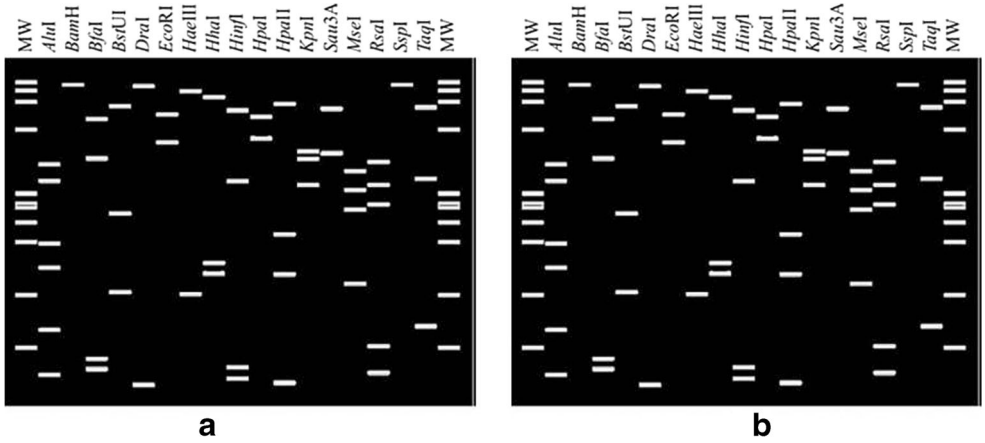

Fig. 4 Comparison of virtual RFLP patterns derived from *in silico* digestions of 1.25 kb 16S rDNA sequences of (**a**) sesame phyllody Delhi isolate (KF744233) and (**b**) *H. phycitis* phytoplasma (KF826901) digested using 17 different restriction endonucleases indicating that the sesame and *H. phycitis* phytoplasma belonged to 16Sr I-B phytoplasma subgroup

caged insects and sesame plants after transmission test indicated the presence of 16SrI-B phytoplasma in transmission materials (both plants and insects) as recorded earlier in the this study (data not shown). The transmission assays results confirmed that HP leafhopper may play a significant role as a natural vector of phytoplasma associated with SP disease.

Discussion

In the present study, the sesame phyllody phytoplasma belonging to '*Candidatus* Phytoplasma asteris' subgroup B has been identified and characterized by RFLP and phylogenetic analysis of 16S rDNA gene sequencing in symptomatic sesame and the weed host, *Sclerocarpus africanus* Jacq. However, earlier studies confirmed that sesame phyllody and witches' broom symptoms reported worldwide were found to be associated with different groups and subgroups of phytoplasmas, viz. 16SrI-B subgroup in Myanmar (Win et al. 2010), 16Sr II-D in Pakistan, Oman and Turkey (Akhtar et al. 2008; Al-Sakeiti et al. 2005; Cengiz et al. 2014), 16SrVI-A, 16Sr IX and 16Sr IX-C subgroup in Turkey (Sertkaya et al. 2007; Catal et al. 2013; Cengiz et al. 2014). In India, so far only 16Sr I group identification was confirmed with sesame phyllody and the characterization of 16Sr I-B subgroup of phytoplasma was reported only with sandal spike (Khan et al. 2008), oil palm (Azadvar et al. 2012), sugarcane, *Brachycome* spp. and *Petunia* hybrida (Madhupriya et al. 2013; 2014) and periwinkle little leaf (Kumar and Byadgi 2012). Hence the association of '*Candidatus* P. asteris' subgroup B with SP, the weed species (SA) and leafhopper (HP) in India is the first report. However, in India the '*Ca* P asteris' group is reported as the major group of phytoplasma affecting a wide host range of plant species

Table 1 Detection of phytoplasma in major leafhopper species collected in sesame fields affected by group 16SrI phytoplasma

Location	Leafhopper species			
	Hishimonus phycitis (Distant)	*Exitinus indicus* (Distant)	*Empoasca prima* (Distant)	*Cofana unimaculata* (Signoret)
Kushinagar				
No. of Individuals	307	189	Nil	177
No. PCR positive /tested samples[a]	12/20	0/20	–	0/20
Phytoplasma group association	16SrI	–	–	–
Delhi				
No. of Individuals	255	163	42	Nil
No. PCR positive /tested samples[a]	8/20	0/20	0/20	–
Phytoplasma group association	16SrI	–	–	–
Gorakhpur				
No. of Individuals	Nil	Nil	135	Nil
No. PCR positive /tested samples[a]	–	–	0/20	–
Phytoplasma group association	–	–	–	–

[a] Number of leafhoppers samples in which phytoplasmas were detected by nested-PCR using universal primers F2n/R2 over the total tested

– not tested

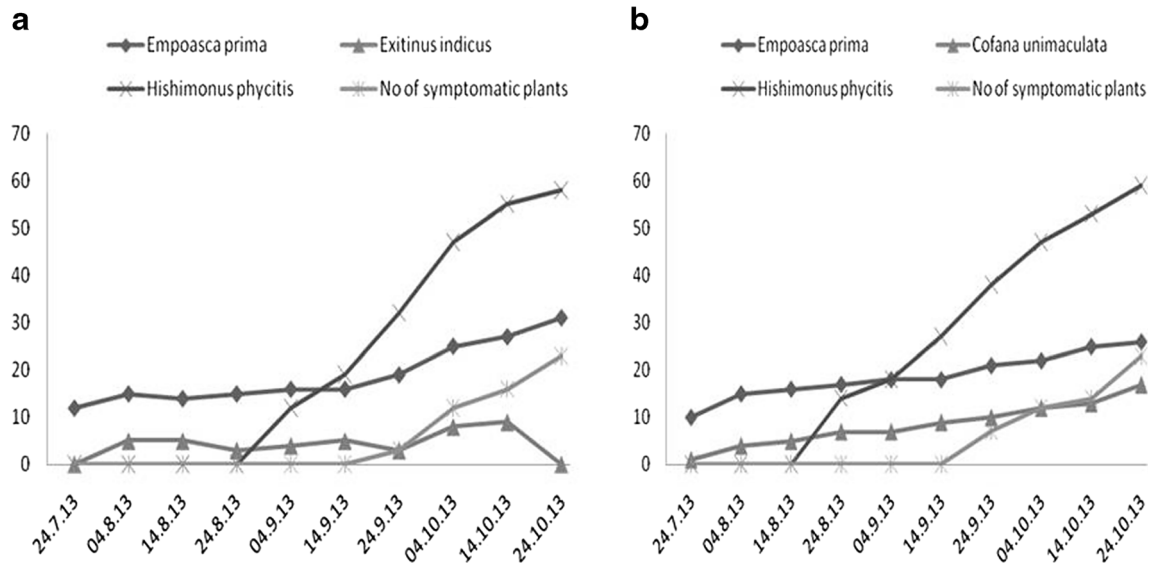

Fig. 5 Population dynamics of leaf hopper species feeding on sesame plants and their correlation with sesame phyllody incidence in the fields (**a**) at Delhi (**b**) at Kushinagar

including agricultural crops, ornamentals, tree and weed species (Rao et al. 2010).

In India, SP transmission was demonstrated by the leafhoppers, *Orosius albicinctus* Distant (Cicadellidae: Hemiptera) (Vasudeva and Sahambi 1955) and *O. argentatus* (Vasudeva 1955) but the causal pathogen of sesame phyllody was considered as virus. Later leafhopper species were recognized as vector for phytoplasmas associated with SP disease in different countries, viz., *Orosius albicinctus* (Distant) (syn: *O. orientalis*) in Iran (Hosseini et al. 2007),Turkey (Sertkaya et al. 2007; Ikten et al. 2011) and Pakistan (Akhtar et al. 2009), *O. cellulosus* Lindberg in Upper Volta (Desmits and Laboucheix 1974), *Circulifer haematoceps* in Turkey (Kersting 1993) and by *Neoliturus haematoceps* in Iran (Salehi and Izadpanah 1992). Though, *O. orientalis* was identified as the major potential vector for SPP in Asia and Africa (Ishihara 1982; Cengiz et al. 2014), in our study, no species of *Orosius* could be observed feeding in sesame fields during the survey period.

Our results suggested that *H. phycitis* (Distant) is a new vector of sesame phyllody phytoplasma (16Sr I-B) in the world. Earlier *H. phycitis* (Distant) leafhopper was reported as a vector of brinjal little leaf phytoplasma belonging to 16SrVI-D subgroup in India (Azadvar and Baranwal 2012) and lime witches broom phytoplasma in Iran in (Siampour et al. 2006; Salehi et al. 2007). In our greenhouse studies, we have successfully demonstrated that SPP belonging to 16Sr-IB subgroup was successfully transmitted from diseased to healthy sesame plants by HP.

The high population densities of HP leafhopper in the present study was also found correlated with increasing incidence of SP symptoms in sesame fields at Delhi and Kushinagar indicates its potentiality for secondary spread of SPP in nature. In the present study, the HP leafhopper population was found increasing from July to September and then decreased from October onwards in sesame fields at Kushinagar and Delhi. The population dynamics was found directly correlated with increase and decrease of sesame phyllody incidence at both the abovementioned places indicating the role of HP as natural vector of SPP in India.

Several weeds are reservoirs of important phytoplasmas which also cause serious diseases to important commercial crops and play an important role in spreading phytoplasmas and serve as natural alternative hosts (Blanche et al. 2003; Pasquini et al. 2007; Mall et al. 2011). Early detection of these phytoplasmas associated with diseases of weed crops is very important to check the possibility of further spread of phytoplasma diseases to other commercial crops. Weed plants could play a key role in the epidemiology of the disease since they influence the population density of the vectors and act as source of inoculum (Pasquini et al. 2007). “Boir noir” (BN) is an important grapevine disease associated with phytoplasma, which is naturally transmitted by the *Hyalesthes obsoletus* vector that complete its life cycle on herbaceous plants specially weeds i.e., *Urtica dioica*; *Calystegia sepium* and *Convolvulus arvensis* (Langer and Maixner 2004; Maixner et al. 2006). In Washington another vector *Circulifer tenellus* (Baker) was reported to transmit Columbia basin potato purple top phytoplasma from weeds to other crops (Munyaneza et al. 2006). *Sclerocarpus africanus* weed species prevalent in sesame fields at Gorakhpur in the present study may also play an important role for natural secondary spread of SP phytoplasma through the leafhopper vector, *H. phycitis*. This report of 16SrI subgroup B on weed, *Sclerocarpus africanus* is the first record of presence of this group of phytoplasma in the world. The result of our study represents the first knowledge about a new

insect vector for sesame phyllody phytoplasma belonging to 16SrI-B subgroup and new weed host species of 16SrI-B phytoplasma in the world. SPP is a major problem in all sesame growing regions in India. The resistant cultivars, management of insect vectors and alternate/collateral hosts would be the most efficient control majors of the disease in the field. Hence the new SPP vector and alternate hosts reported in this study will provide further research prospective on formulating management strategies.

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