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Transmission studies of phytoplasma causing sesamum phyllody

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Abstract
Phyllody is a serious disease of sesame caused by phytoplasma. Different types of disease symptoms are reported to be associated with sesame phyllody. The major symptoms are phyllody (production of leafy structures of floral parts). In the present study investigations were carried out on the transmission and molecular characterization of phytoplasma associated with phyllody. Phyllody disease was successfully transmitted by grafting infected sesame scion to periwinkle stock. The grafted periwinkle plants exhibiting yellowing of leaves and floral virescence was analysed by PCR to confirm the transmission of phytoplasmas from phyllody infected sesame scion to healthy plants. A fragment size of 1200bp was obtained from periwinkle corresponding to the partial 16S rRNA genes. Thus it can be confirmed that transmission of phytoplasma from infected sesame plant to healthy periwinkle was positive. The seeds were collected from phyllody infected sesame plants for seed transmission studies. In an insect proof cage house two hundred seeds were sown in 20 earthen pots. Typical phyllody symptoms were not observed throughout the observation period. It was clearly indicated that the disease is not transmitted through the seeds collected from phyllody infected sesame plants.

Keywords: periwinkle, nested PCR, grafting, primers

Introduction
Sesame (*Sesamum indicum* L.) is under constant threat to many diseases viz., charcoal rot/ stem rot/ root rot, powdery mildew, leaf blight, wilt, leaf spot, stem blight, bacterial leaf spot and phyllody. Among all the diseases the economically important disease affecting sesamum is phyllody, considered to be caused by a virus which was later confirmed as mycoplasma like organisms (MLO), and recently termed as phytoplasmas (Das and Mitra, 1998) [4] which takes a heavy toll resulting in significant yield losses. Phytoplasmas (Mycoplasma - like organisms) are specialized bacteria which do not have cell wall are obligate parasites found in sieve elements of plants and some insect vector. They are transmitted from one plant to another by phloem feeding insects, leafhopper, plant hoppers and psyllids. Yellow disease of plant presumed earlier to be caused by virus, however could not be visualized in affected plants (Lee and Davis 1992) [8]. Japanese scientists were the first to describe phytoplasma as the plant pathogens responsible for yellow disease (Doi *et al*., 1967) [5].
As the phytoplasma cannot be cultivated *in vitro*, rRNA gene sequence revealed that they were non-spiroplasma, wall-less prokaryotes that colonize plant phloem and insect, constitute a group within the class Mollicutes. It is proposed to accommodate phytoplasma within the genus Candidatus phytoplasma. Phytoplasma are surrounded by a single-unit membrane, lack rigid cell wall, pleomorphic in shape with an average diameter of 0.2-0.8µm (Doi *et al*., 1967; McCoy *et al*., 1989) [5, 16] and transmitted by sap sucking insect vectors belonging to the families Cicadellidea (leaf hopper) and Fulgoridea (plant hopper) (Banttari and Zeyea, 1979) [2], vegetative propagation through grafts, cuttings, storage tuber, rhizome or bulb (Lee and Davis, 1992) [8]. However, unlike viruses they are not transmitted mechanically by inoculation with phytoplasma containing sap from affected plants. In host plants they exhibit a range of symptoms as phyllody, floral virescence, sterility of flowers, proliferation of auxiliary shoot resulting in witches‟ broom, generalized stunting, abnormal elongation of internodes resulting in slender shoots.
Phytoplasma have been associated with several diseases in hundreds of plant species belonging to 98 families that include many food, vegetable, fruit crops, ornamental plants and shade trees (Lee *et al*., 2000) [9]. Occurrence of phytoplasma is worldwide and they have been reported in at least 85 countries (McCoy *et al*., 1989) [16]. Phytoplasma do not have a uniform distribution (Lee *et al*., 2000) [9] many are restricted to one continent or to a specific geographical region. In recent years incidence of phyllody is increasing in sesame fields of Telangana state. In view of the seriousness of the disease and scarcity of the related.

information on these phytoplasmas especially on sesamum, the present investigations have been undertaken to study the nature of transmission of sesame phyllody

Material and Methods

The present investigation of phytoplasma disease of sesame (*Sesamum indicum* L.) was carried out at ICAR Indian Institute of Oilseeds Research and Department of Plant Pathology, Professor Jayashankar Telangana State Agricultural University during 2017-18. Studies on transmission and characterization of sesamum phyllody were carried out using standard protocols and presented hereunder.

I. To study the nature of transmission of Sesamum Phyllody through Grafting

1. Graft transmission

Donor plants: Infected plants of sesamum maintained in insect proof glasshouse were used as donor plants for the study.

Method of grafting

Transmission of phytoplasma from plant to plant occurs primarily during the feeding activity of inoculative vector insects, by the vegetative propagation of infected plant material or by graft inoculation. Five plants of periwinkle were used in the study. Side grafting method was employed for graft transmission. The disease scions from sesame infected plants were cut to from both side into 'V' shaped structure. The scions were inserted into the slanting cut made on the healthy stock plants of Periwinkle. The grafted portion was tied tightly with a high density polythene strip. The inoculated plants were kept in insect proof glasshouse for symptom development. Observations were recorded on number of plants showing symptoms and time taken for symptom development. Further the grafted Periwinkle plant showing phyllody symptoms were run for PCR. The DNA was isolated from the healthy and periwinkle plant showing phyllody symptoms and analysed using direct and nested PCR with two sets of primers P1/P7 (Table.1) and R16F2n/R16R2 (Table.1) respectively

2. To study the transmission of phytoplasma from sesame to periwinkle through PCR

Molecular analysis was carried out in periwinkle grafted with Sesamum phyllody to determine the transmission of phytoplasma.

The steps followed for detection and characterization of phytoplasma in periwinkle

1. Preparation of stock solutions
2. Leaf sampling of phyllody infected periwinkle samples.
3. Isolation of genomic DNA
4. Quality and quantity assessment of DNA
5. Polymerase Chain Reaction(PCR)
6. Nested PCR.

1. **Preparation of Stock Solutions:** 1 M Tris HCL, 0.5 M EDTA and 2.5 M NaC
2. **Leaf sampling of periwinkle samples:** Leaf sampling was done by taking 200-250 mg of periwinkle infected leaf samples. Sample was wrapped in aluminum foil labelled and then frozen in liquid nitrogen before storing at -80 ºC.
3. **DNA isolation:** DNA was isolated from the leaf material using CTAB method as per the methodology of Zheng *et al*. (1996) [17]with some modifications

4. Quality and quantity assessment of DNA:

Genomic DNA quantification by agarose gel electrophoresis

Quantification of isolated genomic DNA was performed by running the genomic DNA on 0.8% agarose gel with diluted uncut DNA as standard. Based on the intensity and thickness of genomic DNA bands in comparison to lambda DNA, the concentration and quality of DNA in individual samples was determined.

5. PCR amplification using primers

PCR requirements

1. Template DNA
2. dNTPs: dNTPs wereused.
3. *Taq* DNA polymerase: The *taq* DNA polymerase with 10x *Taq* buffer.
4. Sterile distilled water
5. Thermo cycler eppendorf Master Cyclergradient.
6. Primers used for amplification of 16S rRNA gene of Phytoplasma

Table 1: Primers used for PCR amplification (Sertkaya *et al*., 2007)

	Primer name	Sequence (5-3)
First PCR primer pair	P1-forward primer	AAGAGTTTGATCCTGGCTCAGGATT
	P7-reverse primer	CGTCCTTCATCGGCTCTT
Nested PCR primer pair	R16F2n	ACGACTGCTGCTAAGACTGG
	R16R2	TGACGGGCGGTGTGTACAAACCCCG

Table 2: PCR Programme for amplification of 16S rDNA (first round PCR)

Steps	First Round PCR	
	Periwinkle	
	Temperature(ºC)	Time
Initial denaturation	94	5min
Denaturation	94	45sec
Annealing	55.8	1min
Extension	72	3min
Number of cycles	34	
Final extension	72	10min
Hold	4 ºC	

The volume is in µl/tube. After preparing PCR mixture, the mixture were spun briefly and inserted into the wells of a thermal cycler (Eppendorf Thermo cycler).

Analysis of PCR products by gel electrophoresis

The amplified products were resolved on 0.7% agarose gel (Seakem L Agarose). The gel was run at a constant voltage of 120V for about 45min until the tracking dye migrated to the end of the gel. The gel was observed under UV trans-illuminator and gel documentation system and amplified products were visualized

Nested PCR

The product from direct PCR primed with primers P1 and P7 was diluted 1:80 with sterile deionized water and 1µl was used as template in a Nested PCR.

Table 3: Nested PCR reaction mixture

Reagents	Periwinkle
Sterile distilled water	12.6
10X PCR buffer (supplied with the enzyme)	2.0
2.5mM dNTPs mixture	2.0
R16F2n (5pM)	1.0
R16R2 (5pM)	1.0
Taq polymerase (3u/µl)	0.4
Template DNA (1µl)	1.0

After preparing PCR mixture, the mixture were spun briefly and inserted into the wells of a thermal cycler (Eppendorf Thermocycler).

Analysis of nested PCR products by gel-electrophoresis

One microliter of PCR mixture with 1µl of loading dye (Bromophenol blue) was loaded onto 0.7 per cent agarose gel alongside double digest marker. Electrophoresis was carried out at 120v for 45 min. 1XTAE buffer was used. The DNA bands were visualized on UVtrans illuminator.

Table 4: PCR Programme for amplification of 16S rDNA (Second round PCR)

Steps	Second Round PCR(Nested PCR)	
	Periwinkle	
	Temperature(ºC)	Time
Initial denaturation	94	5min
Denaturation	94	45sec
Annealing	52.2	1min
Extension	72	3min
Number of cycles	34	
Final extension	72	10min
Hold	4ºC	

2. Transmission through seed

Seed transmission provides an effective means of introduction of pathogens especially virus and fungi into crop at an early stage, giving randomized foci of infection throughout the field. To study the spread of pathogens within the growing crop, seed transmission is important. Especially distribution of seed borne virus over long distance may occur due to long or high persistence of viruses of seed.

For seed transmission, two hundred seeds each collected from phyllody infected sesame plants of twenty genotypes *viz.*, 33)INT-135-115, 33) GRT-8368, 31)HT-75, 29)G-TIL-10, 26)SI-1687-1, 19) ES-62, 5) Savithri-DN, 3)IS-113-A,23)SWETHA,3) IS-113-A, 14)DS-5, 16)SI-712, 31)Nirmala, 26) GRT-8392, 24) JLT-408,28) JLT-408,21)DS-5, 32)NIC-3181, 8) Thilothama, 17) RT-351, 5) HT-2 were sown in earthen pots containing steam sterilized soil, sand and compost (2:1:1) mixture and maintained in an insect proof cage house. The plants were observed regularly for the development of symptoms and data on per cent disease incidence and the number of plants showing the typical symptoms was collected.

Results and Discussion

Graft transmission

Studies on the transmission of sesamum phytoplasma from infected plant to healthy periwinkle plants was carried out using five healthy plants of periwinkle by side grafting method (Plate 1). Among five plants of periwinkle grafted with sesamum phyllody only one plant showed positive symptoms of phyllody from donor host Sesamum indicum to receptor periwinkle (Plate 2).

The results indicate that, sesamum phyllody can be transmitted through grafting which developed symptoms within 35 days after grafting. Further, the grafted plants were analysed by PCR assay to confirm the transmission of phytoplasmas from phyllody infected sesame scion to healthy periwinkle stock. The total DNA was extracted from the healthy and infected periwinkle plants using CTAB method.

Total DNA extracts were amplified in a direct PCR assay using primers P1/P7 in the first round amplification. A 1.8kb fragment corresponding to entire 16S rRNA gene plus the 16S-23s spacer region and 5' end of the 23S rDNA were obtained in direct PCR using Universal phytoplasma primers P1/P7 (Fig.1). Further the diluted PJ/P7 (Table 1) products were used as template in Nested PCR (Table 4) with primer pair R16F2n/R16R2. A fragment size of 1.2kb was obtained from periwinkle corresponding to the partial 16S rRNA gene (Fig.2). These results indicate the transmission of phytoplasma from infected sesamum to healthy periwinkle through grafting. Similar observations were also reported by Salehi and Izadpanah (1992) [11] and Akhtar *et al*. (2009) [1]. The phytoplasmas move with in plants through the phloem from source to sink and they are able to pass through sieve tube elements in phloem tissues (Christensen *et al*., 2004) [3]. Due to this nature probably they are transmitted in the process of grafting. Jarausch *et al*. (1999) [6] observed successful transmission of European stone fruit yellows (ESFY) phytoplasma by graft transmission. Kaminska and Korbin (1999) [7] reported graft transmission of phytoplasma affecting Lily plant. Likewise, Salehi *et al*. (2009) [11] could successfully transmit sunflower phyllody phytoplasma by graft transmission.

Seed transmission

The seeds of twenty genotypes of sesamum *viz.*, 33)INT-135-115, 33) GRT- 8368, 31) HT-75, 29) G-TIL-10, 26)SI-1687-1, 19) ES-62, 5) Savithri-DN,3) IS-113-A, 23) SWETHA, 3) IS-113-A, 14) DS-5, 16) SI-712, 31) Nirmala, 26) GRT-8392,24) JLT-408,28) JLT-408,21) DS-5,32) NIC-3181,8) Thilothama,17) RT-351,5) HT-2were collected from phyllody infected sesame plants.

Table 5: Seed transmission of phytoplasma in different genotypes of sesame seeds.

Name of the genotype	Number of seeds sown	Number of seeds germinated	Number of plants showing typical phyllody symptoms	Transmission (%)
33) INT-135-115	20	18	0.00	0.00
33)GRT-8368	20	18	0.00	0.00
31)1 IT-75	20	15	0.00	0.00
29)G-TIL-10	20	16	0.00	0.00
26)SI-1687-1	20	18	0.00	0.00
19)ES-62	20	20	0.00	0.00
5)Savithri-DN	20	20	0.00	0.00
3)IS-113-A	20	16	0.00	0.00
23)SWETHA	20	17	0.00	0.00
3)IS-113-A	20	16	0.00	0.00
14)DS-5	20	16	0.00	0.00
16)SI-712	20	20	0.00	0.00
31)Nirmala	20	20	0.00	0.00

26)GRT-8392	20	18	0.00	0.00
24)JLT-408	20	18	0.00	0.00
28)JLT-408	20	18	0.00	0.00
21)DS-5	20	19	0.00	0.00
32JNIC-3I81	20	19	0.00	0.00
8) Thilothama	20	17	0.00	0.00
17)RT-351	20	17	0.00	0.00
5)HT-2	20	17	0.00	0.00

Two hundred seeds of each genotype was sown in 20 earthen pots containing steam sterilized soil, sand and compost (2:1:1) mixture and maintained in an insect proof cage house. The plants were observed for the expression of symptoms on sesamumplants. However, none of the plants produced any symptoms throughout the period of study (Plate 3). The results clearly indicated that the disease is not seed transmitted confirming the earlier reports of Shankar *et al.*, 2017 [14]; Ravinder, 2017 [10]; Tan, 2010 [15]; Akhtar *et al.*, 2009 [1].

In the present study the experiment was conducted under glass house in insect proof cages indicating that the disease is vector transmitted but not through seed.

Plate 1: Side grafting of infected sesame scion on healthy periwinkle stock

Plate 2: Floral virescence symptom on graft inoculated periwinkle plant

Plate 3: Seed transmission of phytoplasma in different genotypes of sesamum

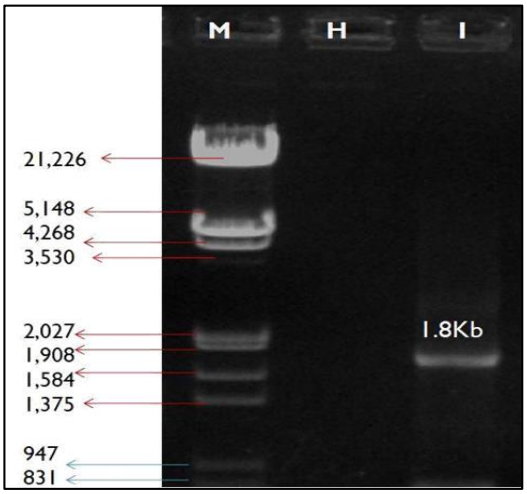

Fig 1: PCR analysis of the phytoplasma from healthy and diseased periwinkle plants with P1/P7 primers **M -** Hind III digested lambda DNA **H** - Healthy periwinkle sample **I** - Infected periwinkle sample

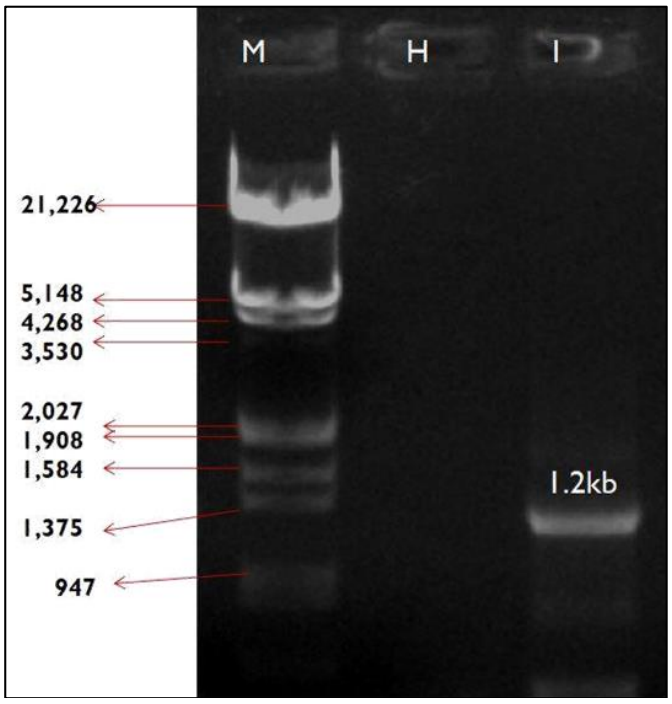

Fig 2: Nested PCR analysis of the phytoplasma from healthy and diseased periwinkle plants with R16F2n/R16R2 primers M - Hind III digested lambda DNA H - Healthy periwinkle sample I - Infected periwinkle sample

Conclusion

The graft transmission of sesamum phyllody was carried out in five periwinkle plants by side grafting method. Among five plants only one plant showed phyllody symptoms indicating that the disease can be transmitted through grafting. The grafted periwinkle plants exhibiting yellowing of leaves and floral virescence was analysed by PCR to confirm the transmission of phytoplasmas from phyllody infected sesame scion to healthy plants. A fragment size of 1200bp was obtained from periwinkle corresponding to the partial 16S rRNA genes. Thus it can be confirmed that transmission of phytoplasma from infected sesame plant to healthy periwinkle was positive, though it needs lot of elucidation.

To ascertain the transmissibility of disease from infected seed to subsequent generation an experiment was conducted by collecting the seeds from the naturally infected with the phyllody symptomatic sesamum plants of twenty genotypes sown earlier. The experiment was carried out in insect proof cages under glass house conditions to prevent the vector. All the twenty genotypes raised from the seed of symptomatic plants did not exhibit any phyllody symptoms in the subsequent generation and the plants were disease free. Thus the results indicated that the sesamum phyllody is not seed transmitted through seed.

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