

Sesame phyllody disease: its symptomatology, etiology, and transmission in Pakistan

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Abstract: Phyllody is a serious disease of sesame in Pakistan. In the present study investigations were carried out on the symptomatology, etiology, and transmission of this disease. Floral virescence, phyllody, and proliferation are the most common symptoms. Sometimes these symptoms are accompanied by yellowing, cracking of seed capsules, germination of seeds in capsules, and formation of dark exudates on the foliage. Shoot apex fasciation has also been occasionally observed, but no phytoplasma DNA has been detected in fasciated plants using PCR assays. Light microscopy of hand-cut sections treated with Dienes' stain showed blue areas in the phloem region of phyllody-infected plants. Pleomorphic bodies (phytoplasma structures) were observed in phloem sieve elements in diseased plants using transmission electron microscopy (TEM). Amplification of a phytoplasma characteristic 1250-bp 16S rDNA fragment confirmed that sesame was infected by a phytoplasma, and RFLP profiling and sequencing confirmed that the associated phytoplasma had the greatest homology to 16SrII-D group phytoplasmas. Phyllody disease was successfully transmitted by grafting dodder (*Cuscuta compestris*) and leafhopper (*Orosius albicinctus*). Treatment of infected plants with tetracycline-HCl provided temporary recovery from the disease.

Key words: *Sesamum indicum*, phyllody, symptomatology, etiology, transmission, Pakistan

Introduction

Sesame (*Sesamum indicum* L.), which originated in Africa, is probably the most ancient oil seed plant cultivated in many parts of the world. Currently, China, India, and Myanmar (Burma) are the world's largest producers of sesame, followed by Sudan, Nigeria, Pakistan, Bangladesh, Ethiopia, Thailand,

Turkey, and Mexico (FAO, 2004). Sesame seed is a rich source of protein (20%) and edible oil (50%), and contains about 47% oleic acid and 39% linolenic acid (Shyu and Hwang, 2002). Sesame oil has excellent stability due to the presence of the natural antioxidants sesamoline, sesamin, and sesamol. Oil from sesame seeds is used in cooking, salad

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preparation, margarine, and raw materials for the production of some industrial materials, including paints, varnishes, soaps, perfumes, pharmaceuticals, and insecticides, while sesame seeds are used in baking, candy, and in other food industries. Seeds with hulls are rich in calcium (1.3%) and provide a valuable source of minerals. The addition of sesame to the high-lysine meal of soybean makes a well-balanced animal food (Jin et al., 2001).

Although sesame is widely used for different purposes, the crop has low yield capacity compared to other plants due to its low harvest index, susceptibility to diseases, seed shattering, and indeterminate growth habit (Ashri, 1998). Among the major constraints, phyllody is a very serious disease in most sesame growing regions and dramatically decreases sesame yields, especially in warm climates (Salehi and Izadpanah, 1992). McGibbon (1924) was the first to report its occurrence in Burma. The disease has now been recorded in India, Iran, Iraq, Israel, Burma, Sudan, Nigeria, Tanzania, Pakistan, Ethiopia, Thailand, Turkey, Uganda, Upper Volta, and Mexico. It has also been referred to as “green flowering disease” or “Pothe” in Burma, “sepaloidy” and “stenosis” in India, and “phyllomania” or “green flowering” in Africa.

Phyllody of sesame, sometimes erroneously called “leaf curl”, was first recorded in the Indo-Pakistan subcontinent at Mirpur Khas (Sindh Province of Pakistan) in 1908 (Vasudeva and Sahambi, 1955; Vasudeva, 1961). According to Vasudeva (1961), a diseased specimen collected from Mirpur Khas on 15 October 1908 is still preserved at the herbarium in New Delhi (India). Although the disease has been known to occur in Pakistan for many years, the causal agent, exact symptomatology, and transmission properties have not been described in detail. Preliminary results on the identification of the causal agent as a phytoplasma have been reported (Akhtar et al., 2008).

Herein we describe the disease symptoms, provide further details on the phytoplasma associated with the disease, and provide evidence for its means of transmission in Pakistan.

Materials and methods

Symptomatology

Observations on phyllody disease of sesame began 1 week after germination at the Nuclear Institute for Agriculture and Biology (NIAB), Faisalabad, Pakistan, between 2004 and 2007. Both symptomatic and asymptomatic plants were tagged in the naturally infected fields at different growth stages. These plants were examined for the main and distinguishing features, as suggested by Vasudeva (1961), Bos (1970), Salehi and Izadpanah (1992), Kersting (1993), and Nakashima et al. (1999).

Etiology

Light microscopy

Tissue sections about 1-2 mm long were cut with razor blades from healthy and infected plant samples, and fixed in a fixative solution at pH 7.4 for 2 days at 4 °C, as described by Neinhuis et al. (1982). After 2 days, free-hand cut transverse sections were stained for 10 min in a 0.2% solution of Dienes' stain at 30 °C, according to Deeley et al. (1979).

Transmission electron microscopy

Water agar-embedded healthy and phyllody infected sesame plant samples were pre-fixed in 5% glutaraldehyde overnight, washed with 0.2 M Pipes buffer, and post-fixed in 1% osmium tetroxide for 18 h at room temperature. The samples were washed with distilled water, treated with 5% uranyl acetate for 16-18 h, and washed again with distilled water. These were then dehydrated with absolute ethanol and embedded in Spur resin over a period of 1-2 days to achieve maximum resin infiltration. The samples were polymerized in pure Spur resin in moulds incubated at 70 °C for 48 h. The polymerized resin blocks were hand trimmed and ultra-thin serial sections 120 nm thick were cut on an RMC MT 7000 ultra-microtome. The sections were put on copper grids, and double stained with 5% uranyl acetate for 30 min and lead citrate for 10 min. Observations were made at the National Institute for Biotechnology and Genetic Engineering (NIBGE), Faisalabad, Pakistan, with a JEOL JEM1010 transmission electron microscope operating at 80 kV.

Molecular characterization

To identify the phytoplasma associated with the disease, DNA was extracted from 300-500 mg of leaf tissue collected from symptomatic and asymptomatic plants using the cetyl trimethyl ammonium bromide (CTAB) method of Doyle and Doyle (1990). Amplification of the 16S rRNA gene was performed in 25 μ L reactions for all samples, using illustra PuRe Taq Ready-To-GoTM PCR beads (Amersham Pharmacia Biotech, Amersham, UK), and 15 ng of template DNA and 100 ng of each primer in an MJ Research PTC200 thermocycler. The phytoplasma universal primer pairs P1/P7 and R16F2/R16R2 were used for the first and second PCR rounds, respectively, under conditions previously described (Hodgetts et al., 2007). Following PCR, 5 μ L of PCR product was separated on 1.2% agarose gel in 1 \times TBE buffer containing ethidium bromide and visualized under UV light. For RFLP analysis, restriction endonuclease digestion with *Hae*III, *Rsa*I, and *Alu*I on the nested PCR products was performed according to the manufacturer's instructions in a 10 μ L final volume, and results were compared using RFLP digestion of faba bean phyllody (Acc. No EF193355) and Australian tomato big bud (Acc. No EF193359) DNA obtained from the collection of the University of Nottingham, UK. Sequencing of PCR products (from primers R16F2n to rU3) (Lorenz et al., 1995) was performed using Beckman Quickstart kit technology and a CEQ 8000 Genetic Analysis System at the University of Nottingham School of Biosciences Sequencing Centre. BLAST searches (Altschul et al., 1990) were performed at the National Center for Biotechnology Information (NCBI) web site (<http://www.ncbi.nlm.nih.gov/>). Phylogenetic trees were constructed with partial 16Sr sequences obtained between primers R16F2n and rU3. Sequence alignment was performed using ClustalW (Thompson et al., 1994). Phylogenetic and molecular evolutionary analyses were performed using the MEGA v.3.1 (Kumar et al., 2004) neighbor-joining method and all default values, with 1000 replications for bootstrap analysis.

Transmission studies

Seed transmission

One hundred seeds harvested from sesame plants infected with phyllody disease were planted in pots under insect-free conditions in a greenhouse. Plants raised from these seeds were observed for symptom development until maturity.

Sap inoculation

Sesame plant tissues with typical phyllody disease symptoms were collected and ground in 0.02 M phosphate buffer (pH 7.4; 1 g mL⁻¹) with a mortar and pestle, and then squeezed through very fine muslin cloth. Young leaves from ten 4-week-old healthy sesame plants were dusted with 500-mesh carborandum powder and mechanically inoculated with the freshly extracted sap using cotton pads. Plants were rinsed with a gentle stream of water immediately after inoculation to remove superfluous inoculum and placed in insect-free cages for symptom development.

Graft inoculation

Ten 4-week-old sesame plants were graft inoculated using phytoplasma inoculum under greenhouse conditions. For grafting, a sliced cut was made on the stem 2 cm below the tip. A 13-cm long sesame branch exhibiting typical phyllody symptoms was detached from an infected plant and a similar cut (as on the test plant) was made on this branch. The corresponding cut surfaces were tied together with parafilm. The scion was dipped into a test tube containing distilled water. Distilled water was changed daily and after 7 days the tubes were removed. Grafted plants were observed daily for symptom development.

Dodder transmission

Dodder (*Cuscuta compestris*) strands were established on phyllody disease-infected sesame plants for 4 weeks. The newly developed dodder strands from diseased plants were then transferred to 5-week-old healthy sesame seedlings. The latter plants were freed of dodder after 4 weeks and observed for symptom development.

Leafhopper transmission

Three leafhopper species, *Orosius albicinctus* Distant, *Empoasca* spp., and *Circulifer* spp., were found in fields with a high incidence of phyllody disease. A group of 25 adult leafhoppers per species first fed on diseased plants for 7 days for disease acquisition. The same leafhoppers were then released onto 10 caged healthy plants (4-weeks-old) for an inoculation period of 7 days. Leafhoppers were then killed and the test plants were monitored daily for symptom development.

Effect of antibiotic treatment on sesame phyllody

A set of 10 phyllody disease-infected sesame plants of uniform size were sprayed 3 times with tetracycline-HCl (500 ppm) at weekly intervals. Plants sprayed with distilled water served as controls.

Results

Symptomatology

Different types of phyllody disease symptoms were observed on sesame plants. The major disease symptoms were floral virescence (Figure 1), phyllody (Figure 2), and proliferation (Figure 3). Additionally, seed capsule cracking (Figure 4), seeds germinating in capsules (Figure 4), formation of dark exudates on foliage and floral parts (Figure 5), and yellowing (Figure 6) sometimes accompanied the disease. Shoot apex fasciation (Figure 7) was also observed on occasion. Phyllody infected sesame plants exhibited symptoms that varied according to growth stage and time of infection. Infection at an early stage of growth resulted in cessation of internode elongation, reduction in leaf size, and stunting (to about two-thirds of normal plant height). The entire inflorescence was converted into twisted reduced leaves closely arranged on the top of the stem, with very short internodes (Figure 6). Infections that occurred later in the season caused characteristic symptoms, such as virescence, phyllody, and witches' broom.

The most characteristic symptoms of the disease are transformation of floral parts into green leaf-like structures, followed by abundant vein clearing in different floral parts. The ovary is replaced by



Figure 1. Floral virescence.

elongated structures, almost resembling a shoot (Figures 1 and 2). The calyx becomes polysepalar, and the sepals become leaf-like and remain smaller in size (Figures 1 and 2). Phylloid flowers become actinomorphic in symmetry, and the corolla becomes polypetalous and deep green. The veins of the flower become thick and quite conspicuous. The stamens retain their shape, but become flattened, showing a tendency to be leaf-like. The anthers become green and contain abnormal pollen grains. The carpals are transformed into a leaf fusion at the margins, and this false ovary enlarges and flattens, exhibiting a soft texture and a wrinkled surface due to the thickening of capillary wall veins. Instead of ovules inside the ovary, there are small petiole-like outgrowths, which later grow and burst through the walls of the false ovary, providing small shoots (Figure 1). These shoots continue to grow and produce more leaves and phylloid flowers (Figure 2). The stalks of the phylloid



Figure 2. Phyllody symptoms.



Figure 3. Floral proliferation.

flowers are generally elongated, whereas normal flowers have very short pedicels (Figure 1).

The severity of the transformation of floral parts into green leaf-like structures was associated with the time of infection. Plants infected before flowering had



Figure 4. Germination of seeds in cracked capsules.

severe symptoms on the entire plant, while plants infected during flowering had severe symptoms on the upper part of the plant, occasionally followed by some rudimentary flowers that yielded very small capsules with degenerated seeds. Sometimes capsules that had set prior to infection cracked longitudinally. The seeds might germinate in such capsules, resulting in hundreds of small shoots. Black exudates on leaves and stems, and yellowing often, but not always, accompanied the disease. Leaves on the lower parts of infected plants, stems, and roots did not exhibit any visible symptoms.

Sesame plants with symptoms of shoot apex fasciation (SAF) were also observed. Symptoms of SAF included flattening of the shoot apex, shortened internodes, and intense proliferation of leaf and flower buds. Flowers of these plants appeared normal in



Figure 5. Dark exudates on foliage floral parts.



Figure 6. Short internodes with yellow, twisted, reduced leaves.

shape, but were rather small and aggregated as clusters at the apex (Figure 7); however, no fasciated sesame samples tested positive for phytoplasma using DNA-based diagnostic techniques.

Etiology

Light microscopy

Under light microscopy regularly distributed dark blue areas were observed in the phloem region of free-hand cut sections from infected plants after Dienes' staining; however, no such areas were observed in similarly prepared sections from healthy tissues. Other than the phloem, no color differentiation was observed in tissues from diseased sesame plants.

Transmission electron microscopy

Electron microscopic studies revealed numerous pleomorphic bodies (phytoplasma) in the sieve elements of xylem cells, phloem parenchyma cells, and companion cells of infected plants, which were absent in healthy plants. These bodies were mostly

spherical to oval, with opaque, low electron density cytoplasm that contained ribosome-like granules and DNA strand-like structures (Figure 8).

Molecular characterization

Nested PCR results using the universal phytoplasma PCR primers P1/P7, followed by R16F2n/R16R2, resulted in products of the expected size (1250 bp) for all infected plant samples, but not for healthy plants (results not shown). Digestion of these PCR products with *Hae*III, *Rsa*I, and *Alu*I, and comparison with the profiles for 16SrII phytoplasmas faba bean phyllody (Acc. No EF193355) and Australian tomato big bud (Acc. No EF193359) indicated that the phytoplasma belonged to the 16SrII group. Sequencing of one clone between primers R16F2n and rU3 confirmed that the phytoplasma had > 99% sequence identity with the 16SrII group sesame phyllody from Oman (Acc. No EU072505) and phylogenetic analysis confirmed that the phytoplasma was a member of the 16SrII-D subgroup (Figure 9).



Figure 7. Shoot apex fasciation.



Figure 8. Electron micrograph showing phytoplasma colonizing a phloem of an infected stem.

Transmission studies

Seed and sap transmission of the infectious agent could not be achieved under greenhouse conditions, which indicates that sesame phyllody is not mechanically or seed transmissible; however, the phytoplasma that causes phyllody disease was successfully transmitted from infected to healthy plants via grafting, dodder, and the leafhopper *O. albicinctus*. The causative agent was successfully transmitted to 10 healthy plants, producing disease symptoms within 25-35 days in all the grafts. Disease transmission in the case of dodder occurred in only 20% of the samples. The leafhopper *O. albicinctus* successfully transmitted the phytoplasma from infected sesame plants to 60% of healthy plants (Figure 10), while *Empoasca* spp. and *Circulifer* spp. failed to transmit the phytoplasma that causes phyllody disease.

Tetracycline treatment

Infected sesame plants sprayed with tetracycline-HCl partially recovered from the typical symptoms of the disease after 20-25 days of treatment. However, all symptoms of the disease re-appeared on new branches 45-55 days after tetracycline treatment.

Discussion

The characteristic symptoms of phyllody disease observed in Pakistan (virescence, phyllody, witches' broom, and stunting) were similar to the symptoms previously described in India (Pal and Pushkarnath, 1935), Thailand (Choopanya, 1973), Israel (Klein, 1977), Iran (Salehi and Izadpanah, 1992), Korea, and Turkey (Kersting, 1993). Under the conditions of the present study, some minor symptoms were observed, such as foliar yellowing, seed capsule cracking, germination of seeds in capsules, and formation of dark exudates, in addition to previously noted symptoms. The presence of dark exudates on the foliage and foliar yellowing requires further investigation. Salehi and Izadpanah (1992) reported that production of dark exudates on foliage in Iran might be due to mixed infection of the phyllody agent with sugar beet curly top virus. In Turkey Baspinar et al. (1993) reported that foliar yellowing of sesame is often caused by a concurrent infection of the sesame phyllody agent plus *Spiroplasma citri*. Plants with

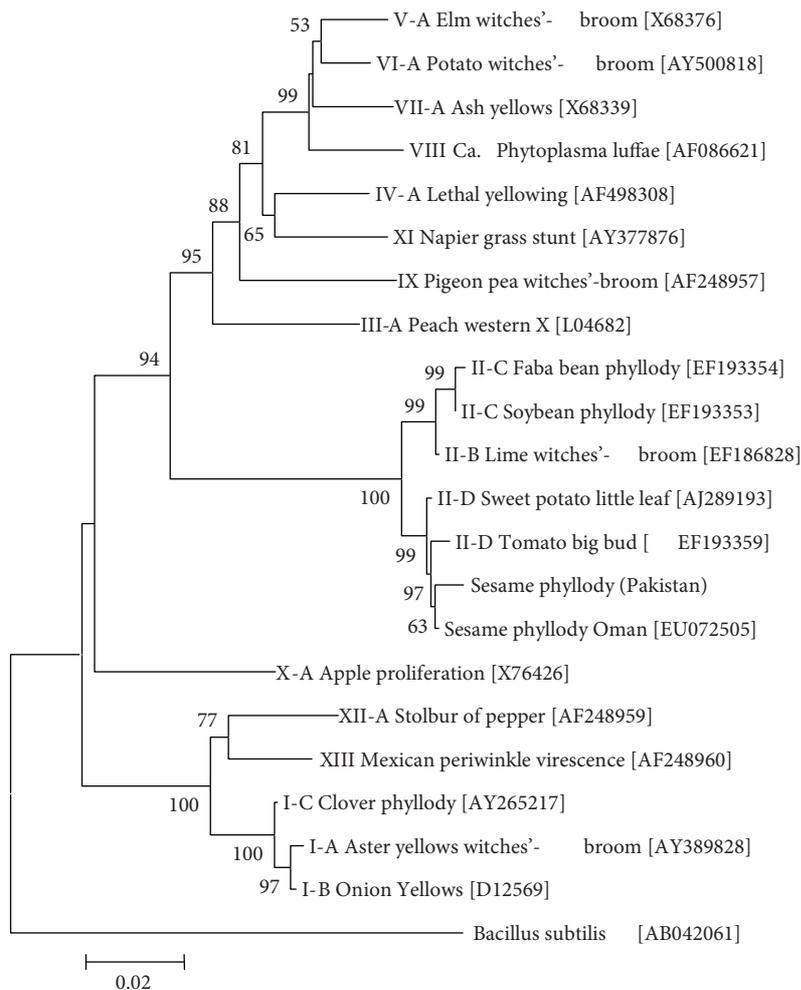


Figure 9. Dendrogram constructed using the neighbor-joining method shows the phylogenetic relationships for partial 16S rRNA sequences (primers R16F2n/rU3) of sesame phyllody (Pakistan), compared with representatives from other 16S rRNA groups. GenBank accession numbers for previously published sequences are shown in parentheses alongside the names of the isolates. Bootstrap values > 50% (expressed as percentages of 1000 replications) are shown, and branch lengths are proportional to the number of inferred character state transformations. Bar: Substitutions per base.

shoot apex fasciation were observed in Pakistan, but no phytoplasma was detected in fasciated plants using molecular techniques. Wilson et al. (2001) similarly found that fasciation in sesame was never associated with phytoplasma infection. In contrast, Tamimi et al. (1989) recorded some pleomorphic bodies in fasciated sesame plants using TEM. The possibility that the bacterium *Rhodococcus fasciens* is associated with the production of fasciation in sesame requires further investigation, as suggested by Wilson et al. (2001).

Phyllody disease of sesame has been recorded in South Asia since 1908 (Vasudeva and Sahambi, 1955; Vasudeva, 1961). Until recently, this syndrome had been classified as a phytoplasma disease, purely on the basis of symptomology (Akhtar et al., 2008). With the present study we confirmed that the disease in Pakistan is caused by a 16SrII-D phytoplasma, based on a positive reaction to Dienes' stain, the presence of pleomorphic bodies in sieve elements (based on TEM), molecular diagnostics, and partial recovery in response to tetracycline-HCl treatment.



Figure 10. *O. albicinctus* (vector).

Dienes' staining in our study showed regularly distributed areas in the phloem region of infected samples, similar to those previously described by Salehi and Izadpanah (1992) in Iran. The current TEM studies revealed the presence of pleomorphic bodies (phytoplasma structures) similar to previously reported phytoplasmas (Salehi and Izadpanah, 1992; Credi, 1994; Samad et al., 2002; Ajayakumar et al., 2007). Amplification of a phytoplasma characteristic

1250-bp 16S rRNA fragment, followed by RFLP analysis and sequencing indicated that the phytoplasma associated with sesame phyllody in Pakistan belonged to the 16SrII-D group ('Candidatus *Phytoplasma australasiae*') and had > 99% sequence homology with sesame phyllody phytoplasma from Oman (Acc. No EU072505), as earlier recorded by Al-Sakeiti et al. (2005).

In the present study phyllody disease was successfully transmitted from diseased to healthy sesame plants using grafting, dodder, and the leafhopper *O. albicinctus*. The disease was previously observed to be vectored by *O. albicinctus* in India (Kolte, 1985; Srinivasulu and Narayanasamy, 1995) and Iran (Esmailzadeh-Hosseini et al., 2007), by *O. cellulosus* Lindberg in Upper Volta (Desmits and Laboucheix, 1974), and by *Neoliticus haematoceps* forma *opacipennis* (J. Dlabola, pers. comm.) in Iran (Salehi and Izadpanah, 1992). Phyllody has also been transmitted from diseased sesame to *Catharanthus roseus* L. by *Circulifer haematoceps* (M. & R.) in Turkey (Kersting, 1993).

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