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Molecular and Electron Microscope Evidence for an Association of Phytoplasma with Sesame Phyllody in Egypt

Om-Hashem M. El-Banna*; M.S. Mikhail*; A.K. El-Attar and A.A.R. Aljamali*****

* *Plant Pathol. Dept., Fac. Agric., Cairo Univ., Egypt.*
** *Plant Pathol. Res. Inst., A.R.C., Giza, Egypt.*
*** *Plant Production. Dept., Fac. Agric., Ibb Univ., Yemen.*

Phyllody is a serious disease of sesame worldwide. In the present study investigations were carried out on the symptomatology, etiology, and transmission of this disease. Floral virescence, phyllody, witches-broom and proliferation are the most observed symptoms on naturally infected plants. In some cases, 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. The plant samples were collected from several fields located in Abou-Rowash County, Giza Governorate. The phytoplasma under investigation was transmitted from diseased plants to healthy ones by grafting with percentage of 100% and by dodder (*Cuscuta compestris*) 20% but not by sap or seeds. Pleomorphic bodies typical to phytoplasma structures were observed in phloem sieve elements in ultra-thin sections of infected plants inspected using transmission electron microscope (TEM). Amplification of a fragment (1250-bp) of 16S rDNA characteristic to phytoplasma using the primer pairs R16F2n/ R16R2 confirmed that sesame was infected by a phytoplasma, Using the primer pair SR1/ SR2 a fragment of 325bp was amplified through nested PCR indicating that the phytoplasma under investigation belongs to witches broom group.

Keywords: Etiology, PCR, phyllody, *Sesamum indicum*, symptomatology, TEM and transmission.

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 (Anonymous, 2011). The total area that is used for growing with sesame crop in Egypt reached 78 thousand feddans and the total production reached 43 tons, (according to statistics of Ministry of Agriculture and Land Reclamation 2011). Sesame seed is a rich source of protein (20%) and edible oil (50%), which 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.

Although sesame is widely used for different purposes, the crop has low yield capacity compared to other crops 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. Thereafter, the disease has been recorded in India, Iran, Iraq, Israel, Burma, Sudan, Nigeria, Tanzania, Pakistan, Ethiopia, Thailand, Turkey, Uganda, Upper Volta and Mexico. Also, it has been got different names, *i.e.* "green flowering disease" or "pothe" in Burma, "sepaloidy" and "stenosis" in India and "phyllomania" or "green flowering" in Africa.

Phytoplasma is a wall-less uncultivable microorganism belong to class Mollicutes. Phytoplasma was transmitted by grafting, insects (specially leaf hoppers). It was transmitted naturally and experimentally by dodder (El-Banna *et al*., 2007 and Mikhail *et al*., 2012).

Because phytoplasma is not cultivated in nutrient media, the methods of its detection and diagnosis are restricted. So electron microscopy is used to check the presence of phytoplasma units in phloem tissues of infected plants (El-Banna *et al.,* 2000 and Rocchetta *et al.,* 2007). PCR based methods are also used for detection and identification of phytoplasma (El-Banna *et al.,* 2007; Samuitiene *et al.,* 2007 and Mikhail *et al.,* 2012).

In Egypt the phyllody symptoms were described in the 1980s but without any deep study about the cause of the disease or its development. So this study was carried out to recognize the disease agent, the association of phytoplasma with the disease and how it is distributed in the field.

Materials and Methods

Symptomatology:

Observations on phyllody disease of sesame began 1 week after germination of sesame seeds at Abou-Rowash County, Giza Governorate, during 2010 and 2011 growing seasons. 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 symptoms characteristic to phytoplasma infection.

Etiology:

Transmission electron microscopy:

Transmission electron microscopy was carried out to detect phytoplasma units inside the infected tissues of sesame plants.

Preparation of plant tissue for examination with electron microscope:

This research was carried out in Res. Park (FARP), Fac. Agric. Cairo Univ., TEM Lab. Samples were transferred to a separate vial to be fixed in 2.5% glutaraldehyde with 0.1M sodium phosphate buffer (pH 7.4) for an hour. After removing the fixative solution, the tissues were washed in sodium phosphate buffer

three times for 30 min each. After washing, the buffer was pulled out and 1% of osmium-tetroxide (OsO_4) was added to the tube and allowed for 1.5 h at 4°C. After removing the fixative solution, the samples were dehydrated in an ethanol series of 15, 30, 50, 70, 80 and 95% before exposing to 100% for 15 minutes for every step except the step of 100% ethanol, which was repeated twice according to the methodology described by Rocchetta *et al.* (2007). Infiltrate with Spurr's epoxy resin as one large drop into the sample tube every 15 minutes, until at 75% resin overnight. Samples were put into 100% resin for at least a day and then samples were placed into flat capsule moulds before hardening the resin overnight in an oven at 60°C. Samples were then sectioned (90 µm thick) with ultra-microtome (Leica model EM-UC6) mounted on copper grids (400 mish). Sections were stained with 5% uranyl acetate then in 10% lead citrate, and then allowed to dry well. Stained sections were examined by transmission electron microscope JEOL (JEM-1400 TEM) at the candidate magnification. Images were captured using CCD camera model AMT.

Transmission studies:

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 and observed for symptoms development (Akhtar *et al.*, 2009).

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 par film. The scion was dipped into a test tube containing sterilized water. Water was changed daily and after 7 days the tubes were removed. Grafted plants were observed daily for symptoms development (Akhtar *et al.*, 2009).

Dodder transmission:

Dodder (*Cuscuta compestris*) seeds were germinated at lab conditions, and then the strands were established on phyllody disease-infected sesame plants for 4 weeks. The newly developed dodder strands taken from diseased plants were then transferred to 5-week-old healthy sesame seedlings. The latter plants were free of dodder after 4 weeks and observed for symptoms development (El-Banna *et al*., 2007 and Akhtar *et al.*, 2009). Percentage of transmission was calculated.

Seed transmission:

One hundred seeds harvested from sesame plants naturally infected with phyllody disease were planted in pots under insect-free conditions in a greenhouse.

Plants raised from these seeds were observed for symptoms development until maturity (Akhtar *et al*., 2009 and Nipah *et al.*, 2007).

Polymerase chain reaction:

a- Extraction of total nucleic acid:

DNA was extracted from symptomatic and asymptomatic sesame plants collected from different Governorates, from sesame plants experimentally inoculated by dodder transmission and from seeds collected from naturally infected plants. Leaf midribs and whole seeds were used for nucleic acid extraction. Using the procedure described by Ahrens and Seemüller (1992), about 1g of each sample was immersed in liquid nitrogen and ground using a pestle attached to an electrical drill with the extraction buffer (CTAB). The nucleic acid pellet was washed with 80% ethanol, air-dried, suspended in 50 µl of sterile distilled water, and maintained at 20°C until use.

b- PCR Primers and amplification:

The DNA extracted from symptomatic and/or asymptomatic sesame plants and from seeds was used as template for PCR. The Universal phytoplasma-specific primers, illustrated in Table (1) were used to amplify the 16srRNA and 16S/23, spacer region of the phytoplasma genome. The primer pair P1/P7 was used for the amplification of 1.8 kb product of 16s rRNA gene, the spacer region between 16s and 23s rRNA gene, and the start of the 23srRNA gene regions of the phytoplasma genomes (Casati *et al.*, 2010 and Franova *et al*., 2011). One µl DNA extracted from sesame plants was used in fifty microlitres of the PCR mixture in 0.2 ml Eppendorf tube contained the following reaction mixture: 2.5 units of the thermos table Taq polymerase (5µ / µl Promega Corporation USA 0,2 Mm DntpS,5UL of 10X PCR buffer (10 mM Tirs. HCl pH 8.3, 50 mM KCl, 2 Mm mGc l12, 10 NM of each primer R16F2n, R16R2 and sterile deionised water up to 50 µl total volume.

Table 1. Sequences and size and specificity for primers used PCR

Primer	Sequence	Size of the PCR product
P1	AAGAGTTTGATCCTGGCTCAGGATT	1.8 kb
P7	CGTCCTTCATCGGCTCTT	
R16F2n	GAAACGACTGCTAAGACTGG	1.2 kb
R16R2	TGACGGGCGGTGTGTACAAACCCCG	
SR1	AGG CGG ATC CTT GGG GTT AAG TCGTAA	325 bp
SR2	AGG CGA ATT CCG TCCTTCATCGGCTCTT	

The DNA amplification was started with a denaturation step at 94°C for 2 min followed by 35 cycles consisting of denaturation at 94°C for 30 s, annealing at 55°C for 1 min, and primer extension at 72°C for 1.5 min. A final extension step was added for 10 min at 72°C. Reactions were cycled in a thermo cycler (Uno II, Biometra, Germany).

c- Nested-PCR:

To increase the sensitivity of the PCR assay the primer pair R16F2n, R16R2 designed to amplify a portion of 16S rRNA gene (1.2 kb) was used in the nested-PCR as described by (Wang and Hiruki, 2001). One µl of DNA amplified by direct PCR with primer pair P1/P7 from sesame samples were used at 1:10 dilution as template for nested-PCR. The mixture of the nested-PCR reaction (50 µl) was prepared as previously described for the direct PCR. A total of 35 thermal cycles were carried out which included denaturation for 1 min (2 min for first cycle) at 94°C, annealing for 2 min at 50°C, and extension for 3 min (10 min in final cycle) at 72°C.

PCR detection of witches' broom phytoplasma using specific primers:

PCR using witches' broom-specific primers SR1 and SR2 representing the phytoplasma-specific 16S/23S rRNA (rDNA) intergenic spacer region (SR) (Liefting *et al.*, 1996 and Smart *et al.*, 1996), amplification was started with a denaturation step at 94°C for 2 min followed by 5 cycles at 94°C for 15s, 45°C for 15s, and 72°C for 30s followed by 25 cycles of 15s at 94°C, 15s at 55°C and 30s at 72°C, with a final extension of 10min at 72°C.

Electrophoresis analysis:

The amplified DNA was stained with gel star (Lonza, USA) and analyzed on 1% agarose gel with 1xTAE buffer, then photographed using (Gel Doc 2000 Bio.RAD). The molecular weights of the PCR products were determined by comparison with 100 bp and/or 1 kb DNA ladder**.**

Results

Symptomatology:

Different types of phyllody disease symptoms which are the typical characteristics of phytoplasma infection were observed on naturally infected sesame plants. The major disease symptoms were floral virescence (Fig. 1A), phyllody (Fig. 1B), and proliferation (Fig. 1C). Additionally, seed capsule cracking (Fig. 1D), germinated seeds in capsules (Fig. 1D), formation of dark exudates on foliage and floral parts (Fig. 1E), and yellowing (Fig. 1F) are sometimes accompanied to the disease. 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 internodes 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 (Fig. 1F). 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 elongated structures, almost resembling a shoot (Figs. 1A and 1B). The calyx becomes polysepalar, and the sepals become leaf-like and remain smaller in size (Figs. 1A and 1B). Phyllody 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

Fig.1. Symptomatology of naturally infected plants was. (A) Floral virescence, (B) Phyllody, (C) Floral proliferation, (D) Germination of seeds in cracked capsules, (E) Dark exudates on foliage parts and (F) Short internodes with yellow twisted reduced leaflets.

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 (Fig. 1A). These shoots continue to grow and produce more leaves and phyllody flowers (Fig. 1B). The stalks of the phyllody flowers are generally elongated, whereas normal flowers have very short pedicels (Fig. 1A). The severity of the transformation of floral parts into green leaf-like structures was associated with the time of infection. Plants infected before flowering showed severe symptoms on the entire plant, while plants infected during flowering showed 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 may germinate in such capsules, resulting in hundreds of small shoots. Black exudates on leaves and stems and yellowing often, but not always, are accompanied to the disease. Leaves on the lower parts of infected plants, stems and roots did not exhibit any visible symptoms.

Etiology:

Transmission electron microscopy:

Ultrathin sections prepared of leaf petiole tissues of sesame plants representing phyllody symptoms were investigated by TEM, the investigation revealed numerous phytoplasma units in the sieve elements (Fig. 2 A, C). These units are rounded, elongated or pleomorphic, measuring 200 to 400 nm in size, bounded by a unit membrane and lacking cell walls (Fig. 2 B). They contained granules mainly peripheral (ribosomes) centrally located net-like structures (DNA). Phytoplasma units were detected in all the inspected tissues indicating their responsibility of the disease. No phytoplasma units were detected in the inspected tissues of healthy plants (Fig. 2D). No other microorganisms were observed during the investigation of the infected tissues.

Transmission studies:

Sap transmission of the infectious agent could not be achieved under greenhouse conditions, which indicates that sesame phyllody is not mechanically transmissible; however, the phytoplasma that causes phyllody disease was successfully transmitted from infected to healthy plants via grafting and dodder. The causative agent was successfully transmitted to 10 healthy plants, producing disease symptoms within 25-35 days in all the grafts (100% transmission). Disease transmission in the case of dodder occurred in only 20% of the tested plants. Seed transmission of phyllody disease through sesame seeds was not achieved in greenhouse experiment.

PCR based assays:

a. Extraction of total nucleic acid:

Deoxy ribonucleic acids (DNAs) were extracted from symptomatic and healthy sesame plants collected from different Governorates, and from sesame plants experimentally inoculated by dodder transmission. Leaf midribs were used for nucleic acid extraction.

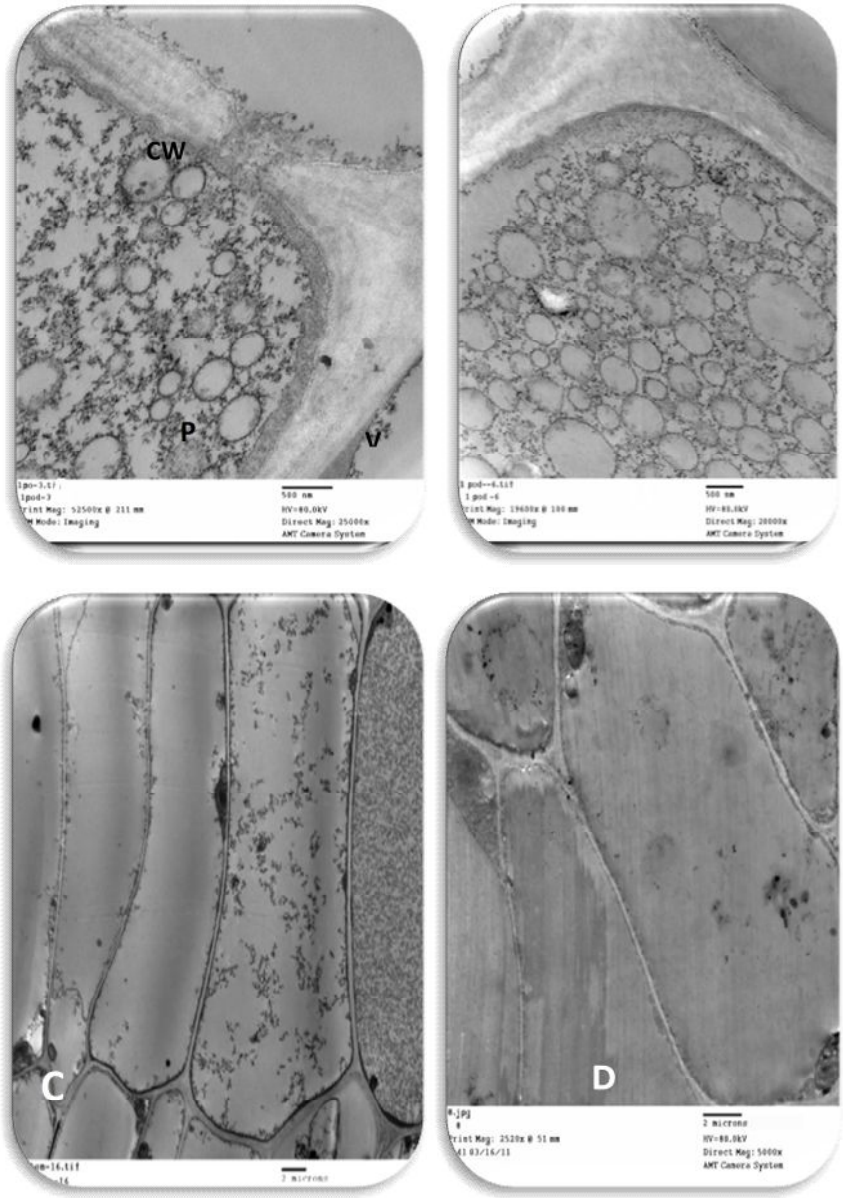

Fig. 2. Electron micrographs showing phytoplasma units (p) colonizing a phloem of an infected plant in different size (A and B x= 20000), (C and D) phloem tissue of healthy plant. x= 3000, cw= cell wall, v= vacuole.

b. Nested PCR:

The universal-phytoplasma specific primer pairs; P1/P7 and R16F2n / R16R2 adopted for detection of the 16S, 23S and the spacer region (SR) fragment of the phytoplasma(s) genome were used for the nested PCR. The intensity of the 1.8 kb fragment for the first round PCR, using P1/P7 primer pair, was too low to be detected by electrophoresis. A fragment of approximately 1.2 kb was amplified by the second round PCR, using R16F2n / R16R2 primer pair, from each DNA sample extracted from infected plants. PCR amplified products were not obtained from healthy plants used as negative controls (Fig. 3). According to the primer pairs P1/P7 and R16F2n/ R16R2, adopted by many investigators (Wang and Hiruki 2001, Salehi *et al*., 2006 and Ribeiro *et al*., 2007), PCR amplification gave products of the expected molecular size, from phytoplasma-infected sesame samples.

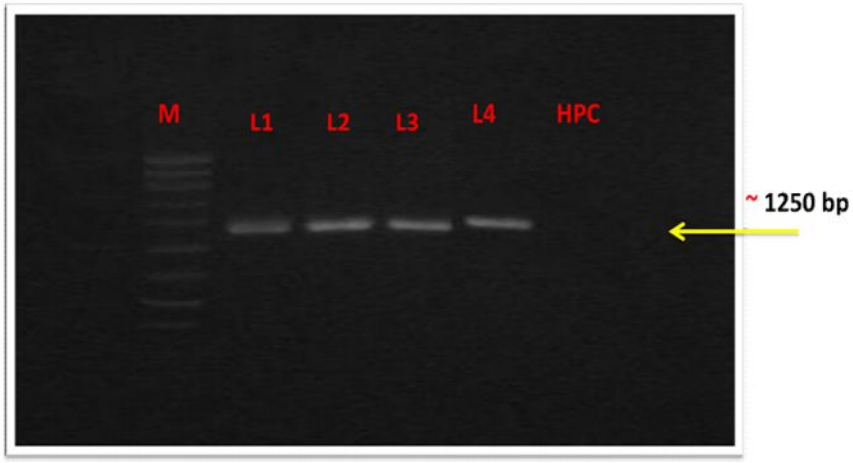

Fig. 3. Gel electrophoresis for the detection of the phytoplasma in sesame using Universal phytoplasma-specific [R16] PCR primers. M: 1 Kb DNA Ladder. L1, L2, L3 and L4 are different samples showed Wiches- broom symptoms. HPC: Healthy plant control.

c. Witches' broom-specific PCR detection:

In the present study nested.PCR assays with the primer pairs P1/P7 and R16F2n/ R16R2 followed by specific detection for the sesame witches' broom using the primer pair SR1/ SR2 yielded a strong band of approximately 325 bp for DNA extracted from sesame representing phyllody symptoms, sesame showing witches'-broom symptoms, sesame plants developing symptoms after dodder transmission, malformed capsules and seeds harvested infected sesame plants (Fig. 4). No visible bands were detected from the corresponding healthy samples. These findings demonstrated the expected association of a phytoplasma with diseased sesame witches broom exhibiting reduction in size leaf proliferation of lateral shoots, and stunting symptoms.

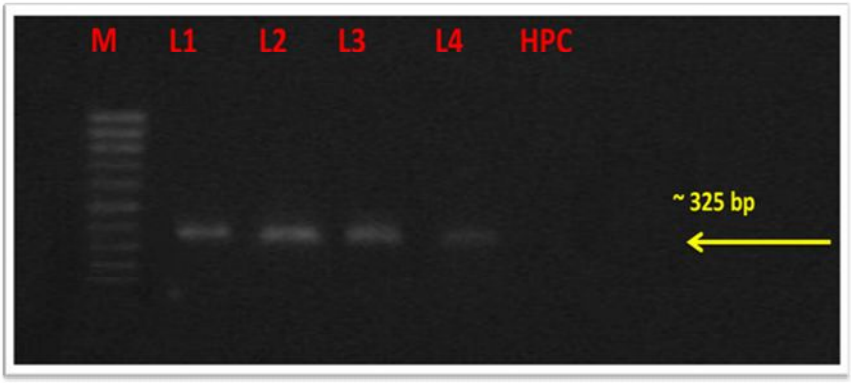

Fig. 4. Gel electrophoresis for the detection of the phytoplasma in sesame using witches' broom-specific PCR primers. M: 100 bp DNA Ladder. L1, L2, L3 and L4 are different samples showed witches–broom symptoms.
Key of samples:

- **L1 : Phyllody for plant infected by dodder.**
- **L2 : Witches' broom on sesame.**
- **L3 : Seeds from infected sesame plant.**
- **L4 : Infected capsules.**
- **HPC: Healthy plant control.**

D i s c u s s i o n

In the present study, phytoplasma isolate was detected in naturally infected sesame plants growing in Abou-Rowash County, Giza Governorate. The plants were characterized by virescence, phyllody, witches- broom, and stunting symptoms .These symptoms 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), Pakistan (Akhtar *et al.*, 2009). In the present study, some minor symptoms were observed, such as foliar yellowing, seed capsule cracking and germination of seeds in capsules and formation of dark exudates, in addition to the 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 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 and Vasudeva, 1961). Until recently, this syndrome had been classified as a phytoplasma disease, purely on the basis of symptomatology (Akhtar *et al.*, 2008). In the present study the authors confirmed that the disease in Egypt is caused by phytoplasma, based on, the presence of pleomorphic bodies in sieve elements (based on TEM), and molecular diagnostics. The current TEM studies revealed the presence of pleomorphic bodies (typical phytoplasma structures) similar to those previously reported by El-Banna and El-Deeb (2007). Phytoplasma units were detected in almost all examined phloem tissues. Phytoplasma units were found in different concentrations (Salehi and Izadpanah, 1992; Credi, 1994; Samad *et al.*, 2002; Ajayakumar *et al*., 2007; El-Banna *et al*., 2007 and Mikhail *et al*., 2012). In the present study, phyllody disease was successfully transmitted from diseased to healthy sesame plants using grafting and dodder indicating the pathogenicity of the detected phytoplasma and verifying its responsibility of sesame phyllody disease. This concept was confirmed by Kolte (1985); Srinivasulu and Narayanasamy (1995), El-Banna *et al.* (2007) and Mikhail *et al.* (2012).

The researchers obtained positive results depending on the nested PCR using the primers specific for detection of witches broom phytoplasma group. So, it could be stated that sesame phytoplasma disease in Egypt belongs to this group.

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دلائل جزيئية وميكروسكوبية على ارتباط الفيتوبلازما بمرض تورق الأزهار على السمسم في مصر

أم هاشم محمد البنا* ، موريس صبري ميخائيل*، أحمد كمال العطار ، أحمد علي راشد الجمالي*****

* قسم أمراض النبات - كلية الزراعة- جامعة القاهرة- رمصر .

** قسم بحوث إمراض الفيرس ـ معهد بحوث إمراض النبات ـ مركز البحوث الزراعية – مصر.

*** قسم الإنتاج النباتي – كلية الزراعة – جامعة إب – اليمن .

مرض تورق الأزهار من أهم أمراض محصول السمسم عالمياً. ولقد أجريت هذه الدراسة للتعرف على طبيعة المسبب المرضي وأعراض الإصابة الظاهرية بالمرض وطرق انتقاله وتعتبر أعراض اخضرار البتلات, وتورق الأزهار ,مكنسة الساحرة وتكشف البراعم من أهم الإعراض الظاهرية للإصابة الطبيعية على النبات. وفي بعض الحالات كانت الإصابة مصحوبة بالاصفرار، وتفتح القرون مع إنبات البذور داخل القرن وتكون إفرازات سوداء، وقد يلاحظ تفلطح في قمة الساق. تم جمع العينات النباتية من مناطق مختلفة هي أبو رواش بمحافظة الجيزة وقنا والإسماعيلية. الفيتوبلازما المشار إليها في الدراسة انتقلت من النباتات المصابة إلى السليمة بالتطعيم بنسبة ١٠٠% وبالحامول بنسبة ٢٠% ولكنها لم تنتقل عن طريق البذور والنقل الميكانيكي. وجدت وحدات الفيتوبلازما متعددة التشكل في قطاعات فائقة الرقة لأنسجة لحاء النباتات المصابة من خلال الفحص بالميكروسكوب الإلكتروني النافذ. وعند تطبيق تقنية PCR تم الكشف عن جزء من 16SrDNA بطول 1250 bp باستخدام بادئ عام R16F2n / R16R2 وتم تأكيد إصابة أنسجة السمسم بالفيتوبلازما التابعة لمجموعة مكنسة الساحرة باستخدام بادئ متخصص SR1/ SR2 على جزء من الحمض النووي عند 325 bp بواسطة Nested PCR وهذا يشير إلى أن الفيتوبلازما ضمن الدراسة تتبع مجموعة مكنسة الساحرة Witches – broom .