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Detection and Control of Rose Phytoplasma Phyllody Disease

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Phytoplasmas causing phyllody symptoms on rose was detected from naturally infected plants. The detected phytoplasma was transmitted by; grafting and dodder into healthy rose and periwinkle plants. Phytoplasma units ranging in diameters from 0.4 to 0.8 µm were detected inside phloem tissues of infected plants. DNA extracted from symptomatic samples was used as template for amplification of products of 1.8 kb using primer pair P1/P7 and 1.2 kb using primer pairs R16F2/R2 by direct and nested-PCR, respectively. Three samples from protected rose fields yielded PCR, amplicons of expected size (1,200 bp) by nested PCR, while no PCR products were obtained for the symptomless plants. Two concentrations of tetracycline hydrochloride, *i.e*. 250 and 300 ppm were used to control the disease by two different treatments, immersing transplants and soil drench. Immersing transplants with tetracycline at 300 ppm for either 15 or 30min gave best result. Also the concentration at 300 ppm for soil drench had a higher recovery effect than at 250 ppm. This study of Rose phyllody disease caused by phytoplasmas is carried out for the first time in Egypt.

Keywords: Electron microscope, PCR, phyllody, phytoplasma, rose and Tetracycline.

Phytoplasmas are wall-less unicellular, phloem-restricted microorganisms of the class Mollicutes. They are transmitted in nature by phloem-sucking vectors (mostly leafhoppers) by dodder and grafting (Salehi *et al*., 2006 and Xiaodong *et al*., 2009). Phytoplasmas are Mollicutes associated with diseases of several plant species including ornamental and medical plant species (Al-Saady and Khan 2006 and Harrison *et al*., 2008) and cause serious economic losses (Chaturvedi *et al*., 2010 and Singh *et al*., 2011). Phytoplasma epidemics have compelled withdrawal of many ornamental varieties from cultivation. General yellowing and stunting of plants, proliferation of shoots, phyllody as well as virescence and reduced size of flowers and reddening of leaves are the common symptoms observed on ornamental plants (Chaturvedi *et al*., 2010). So far, 42 phytoplasmas belonging to 9 groups were identified in ornamental plants worldwide (Chaturvedi *et al*., 2010).Based on the 16Sr sequences identified phytoplasmas in ornamental plants in India mainly belong to 16SrI,16SrII, 16SrIII, 16SrV, 16SrVI, 16SrVII, 16SrIX,16SrX, 16SrXII, 16SrXIII and 16SrXIV groups. Ajaykumar *et al.* (2007), for first time, recorded *Candidatus* phytoplasma asteris' associated with little leaf disease of Portulaca grand flora. Samad *et al.* (2008) reported little leaf disease of Portulaca grand flora at Lucknow.

Raj *et al*. (2009) observed phytoplasma disease in *Chrysanthemum morifolium*, Adeniumobesum and Gladiolus at Lucknow. Chaturvedi *et al*. (2010) reported little leaf disease in *Rosa alba*, *Catharanthus roseus* and Hibiscus *rosa-sinensis* in Gorakhpur. Occurrence, identification, and characterization of phytoplasmas in five further ornamental species in India were reported.

Phytoplasma affect important crops causing a wide variety of general symptoms that ranges from mild yellowing to death of plants. Characteristic symptoms of phytoplasma infections include, virescence, phyllody, witches'-broom, stunting and general decline (Bhat *et al*., 2006). Phytoplasmas are known to cause considerable losses in the yield of ornamental crops (rose, aster, and others) (Torres *et al*., 2004). Traditionally, phytoplasmas were described, identified and differentiated mainly on the basis of their biological properties such as the symptoms they induce, on the host plant affected, and the methods of transmission as grafting and dodder transmission (Suzuki and Oshima, 2006). Phyllody phytoplasma is associated with a wide range of plant diseases worldwide (Padovan and Gibb, 2001; Khan *et al*., 2002 and Xiaodong *et al.,* 2009). Transmission electron microscopy of ultrathin sections of different plant species exhibiting characteristic phytoplasma symptoms were carried out by many investigators (Jomantiene *et al*., 2002; Rita and Favali, 2003 and El-Banna and El-Deeb, 2007). Phytoplasma units are different in size ranging in diameter from 200-400µm and in some cases it reaches 800 µm. They are bounded by one unit membrane; contain ribosomes and the nucleic acid imbedded in the cytoplasm (Ghandi *et al*., 2003; Bhat *et al*., 2006 and El-Banna and El-Deeb, 2007).

Under Egyptian conditions phytoplasma was first detected by El-Banna and El-Deeb (2007) in phloem tissues of mango inflorescences exhibiting mango malformation symptoms. Then on some solanaceous hosts (tomato and pepper) (El-Banna *et al.,* 2007) as in isolate of accession No. EU232714 and EU232715), respectively, was detected and identified

According to the available data, this is the first report of phytoplasma affecting ornamental plants under Egyptian conditions.

The objectives of this study were designed to: 1) Indicate the responsibility of phytoplasma for the diseases by graft and dodder transmission, 2) Verify the presence of phytoplasma in tissues of diseased rose plants using electron microscopy and PCR based techniques and 3) Control the disease by tetracycline application.

Materials and Methods

Plant samples:

Rose (*Rosa* sp.) plants showing typical symptoms of phyllody disease were collected from different fields located in Giza Governorate. The infected plants were uprooted carefully and were potted in 25 cm pots filled with natural soil obtained from the experimental station of Faculty of Agriculture, Cairo University and kept under green house conditions (22-25ºC) as a source for the subsequent studies.

Pathogenicity test:

The pathogenicity of the suspected phytoplasma was verified by both graft and dodder transmission.

a) Grafting:

Naturally infected rose plants served as a source of plant material were used in grafting. Fifteen symptomatic rose plants were used as rootstocks; cleft grafting was carried out with scions taken from healthy rose from the same variety. In all cases the graft unions were kept under mist for three weeks, then kept under greenhouse conditions (22-25°C) and observed for symptoms appearance.

b) Dodder transmission:

In this experiment dodder *(Cuscuta odorata*) seeds were *in vitro* germinated on Petri dishes 12cm diameter bottomed with wetted filter paper for four days at room temperature (22-25°C). The germinated seeds were then transferred onto the stems of naturally infected (symptomatic) rose plants as 3-5 germinated seeds/plant to parasite on them and acquire the pathogen for 16 days. The stolons of the parasitizing dodder were allowed to parasitize on stems of healthy rose, and periwinkle plants to check the transmissibility of the phytoplasma as 12 plants representing each of them were used. The tested plants were kept under green house conditions and observed for symptoms appearance (Back inoculation was carried out by the same method from dodder transmitted phytoplasma into periwinkle plants to confirm the transmission of phytoplasma. In check treatment, dodder stolons were allowed to parasitize on healthy source plants before parasitizing on the tested plants.

Electron microscopy:

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

Preparation of plant tissue for examination with electron microscope:

1- The work was carried out in Cairo Univ., Fac. Agric., Res. Park (FARP), TEM Lab. Samples were transferred to a separate vial to be fixed in 2.5% Glutaraldehyde with 0.1 M 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 (OsO4) was added to the tube and allowed for 1.5 h at 4^{o}C. After removing the fixative solution the samples was 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 repeated twice according to the methodology described by Timothy and Kristen (2000) and Rocchetta *et al.* (2007). Infiltrate with Spurr's epoxy resin, 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^{o}C. Samples were then sectioned (500-1000 µm thick) with ultra-microtome (Leica model EM-UC6), sections were stained with Tolodin blue (1X) then sections were examined by camera Leica (model ICC50 HD).

2- Samples were then sectioned (90 µm thick) with the ultra-microtome mounted on copper grids (400 mesh). Sections were stained with 5% uranyl acetate and lead citrate, and then allowed to dry well.

3- Stained sections were examined by transmission electron microscope JEOL (JEM-1400 TEM) at the candidate magnification. Images were captured using CCD camera model AMT.

Molecular biology studies:

Extraction of total nucleic acid:

Deoxy ribonucleic acids (DNAs) were extracted from symptomatic and healthy rose and periwinkle plants. Leaf midribs were used for nucleic acid extraction. Applying the procedure described by Ahrens and Seemüller (1992) and adopted by El-Banna *et al.* (2007). About 1g of midrib was immersed in liquid nitrogen and ground using a pestle attached to an electrical drill with the extraction buffer (CTAB buffer: 2%, 1.4 M NaCl, 20 mM EDTA, 1% poly vinyl pyrrolidone (PVP), 0.2% mercaptoethanol, 100mM Tris-HCl, pH 8.0). The nucleic acid pellet was washed with 80% ethanol, air-dried, suspended in 50 µl of sterile water, and maintained at -20°C until use.

Primers and PCR amplification:

The DNA extracted from symptomatic rose and periwinkle plants was used as template for PCR. DNA extracted from asymptomatic plants was used as negative controls; the primers illustrated in Table (1) were used to amplify the 16S rRNA and 16S/23, spacer region of the phytoplasmas genome. The primer pair P1/P7 (Sinclair *et al*., 2000 and Bhat *et al*., 2006) was used to prime the amplification of 1.8-kb product of 16S rRNA gene, the spacer region between the 16S and 23S rRNA gene, and the start of the 23SrRNA gene regions of the phytoplasma genomes. For amplification, 1 µl DNA preparation from rose and periwinkle plants were used. Fifty microliters of PCR reaction mix. were added to each Eppendorf tube contained the following reaction mixture 2.5 units of the thermostable *Taq* polymerase (5 u/µl, Promega Corporation, U.S.A.), 2mM dNTPs, 5ul of 10X PCR buffer (10 mM Tris-HCl pH 8.3, 50 mMKCl, 2mM MgCl2, 10µM of each primer P1/ P7 and sterile water to up volume of 50µ.

Table 1. Sequences, size and specificity of the tested primers

Primer	Sequence	Size of the implicated product	Specificity
P1 P7	5'- aagagtttgatcctggct cag gat t -3‘ 5'- cgtccttcatcggctctt -3‘	1.8kb	Universal
R16F2 R16R2	5'- gaaacg act gctaag act tgg-3‘ 5'- tgacgggcggtgtgtacaaac ccc -3‘	1.2kb	Nested aster yellow

The DNA was amplified by 35 cycles consisting of denaturation at 94°C for 30s (2 min. for cycle 1), annealing at 55°C for 1 min, and primer extension at 72°C for 1.5 min (7.5 min. for cycle 35). Reactions were cycled in a thermocycler (Uno II, Biometra, Germany).

Nested-PCR:

To increase the sensitivity of the PCR the primer pair R16F2n/R16R2 designed to amplify a portion of 16S rRNA gene (1.2 kb) was used in the nested-PCR (Wang and Hiruki, 2001 and Samuitiene *et al*., 2007). In the nested-PCR assay, 1 µl of DNA amplified by direct PCR with primer pair P1/P7 from rose samples exhibiting phyllody symptoms was used as template with 1:40 dilution. 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 analysis:

The amplified DNA was electrophoresed on 1% agarose gel with 1xTAE buffer, stained with ethidium bromide and photographed using (Gel Doc 2000 Bio-RAD). The molecular weight of the PCR products were determined by comparison with DNA markers, 100bp ladder (MoBiTec), XIV PCR marker (Roch) and PGEM DNA Markers (Promega).

Control of phyllody diseases with tetracycline hydrochloride:

Two Concentrations of tetracycline hydrochloride, *i.e.* 250 and 300 ppm, were used to control the phytoplasma disease. Two different treatments were applied, *i.e*. soil drench and immersing transplants for 15 and 30 minutes in the candidate concentration. The tetracycline solution was mixed with 5%cetric acid to facilitate the uptake of tetracycline. Treatments included rose and periwinkle plants grown in pots (25-cm-diameter) were experimentally inoculated by dodder transmission and kept for 40 days and showing symptoms of infection by dodder transmission. Percentage of disease recovery was calculated (no symptoms on the new growths formed after treatment).

Results

Diseases symptoms:

The infected rose samples represented phyllody symptoms, malformation of flowers and formation of small green leaves were very obvious. The petals were much reduced in size, excessive and compact growth of flowers was observed compared with healthy roses.

Pathogenicity test:

The responsibility of phytoplasma on the phyllody symptoms was verified by the following methods:

a) Grafting:

Pathogenicity of the detected phytoplasma was checked by grafting into healthy rose plants .The same symptoms phyllody symptoms on rose were obtained on the newly formed plant parts about 45 days after grafting. Percentage of transmission by grafting reached 100% in rose graft unions.

b) Dodder transmission:

The pathogenicity of the detected phytoplasma was also checked by dodder transmission. Dodder stolons transmitted the phytoplasma from naturally infected (symptomatic) rose and periwinkle plants after 12 days of parasitizing on infected plants and after 25 days of parasitizing on the tested plants as the same symptoms were observed on all of them. On the other hand dodder was used in back inoculation of healthy rose plants to confirm the responsibility of phytoplasma for the observed symptoms and the diseases.

Periwinkle plants were also experimentally infected with the phytoplasma by the same method and were kept as a maintaining host. No symptoms were observed on test plants in which stolons of dodder were transferred to them after parasitizing on healthy plants.

Electron Microscopy:

Ultrathin sections prepared of leaf petiole tissues of rose plants representing phyllody symptoms were investigated by TEM, the investigation revealed numerous phytoplasma units in the sieve elements (Fig. 1a). These units were rounded , elongated or pleomorphic , measuring 200 to 400 nm in size, bounded by a unit membrane, and lacking cell walls (Fig. 1b). They contained granules mainly peripheral (ribosomes) centrally located net-like structures (DNA). Phloem parenchyma was almost completely degenerated and contained little or no cytoplasmic residues (Fig. 1c).

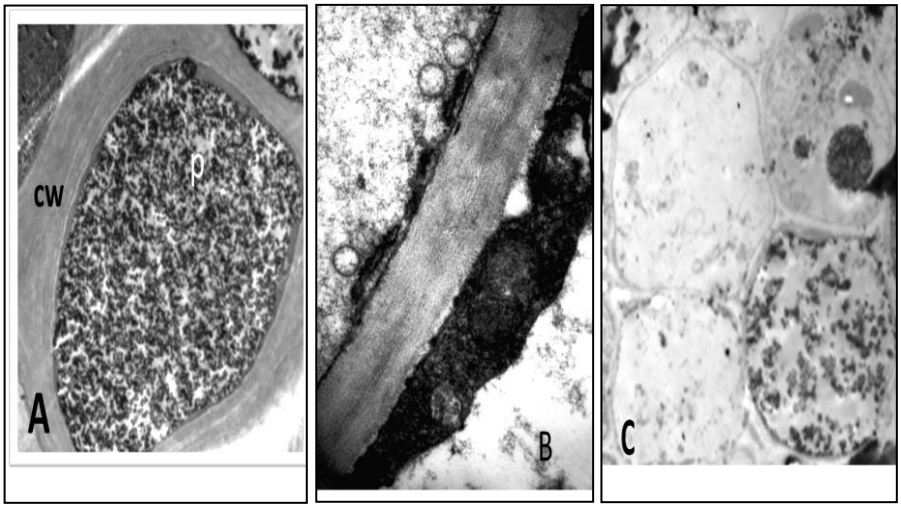

Fig. 1. Transmission electron micrograph of rose phloem tissues infected with phyllody. Whereas: A) One sieve element filled with phytoplasma units (P) thicking of the cell wall (CW) is also obvious (X 5000), B) Phytoplasma units adjacent to the cell membrane of infected rose phloem tissues (X 30000) and C) Phloem parenchyma cell, showing degeneration and lyses of cytoplasm (X 5000).

Molecular biology studies:

Detection of phytoplasma by PCR- based assays:

The universal primer pair P1/P7 was adopted for detection of the 16S, 23S and the spacer region (SR) fragment of the phytoplasma genome (Fig. 2). DNA fragments of approximately 1.8kb were amplified by direct PCR from all total nucleic acid extracted from samples of infected rose and periwinkle plants. PCR amplified products obtained from healthy plants were used as control (Fig. 3). In the present study nested-PCR assays with the primer pair P1/P7 followed by the primer pair R16F2n/R16R2 yielded a strong band of approximately 1.2 kb DNA fragment from DNA extracted from rose and periwinkle plants representing phyllody symptoms after dodder transmission (Figs. 3 & 4). No visible bands were detected from the corresponding healthy samples.

Fig. 2. Map of the rDNA fragment 16S -23S including the spacer region and the primers used for amplification of these regions.

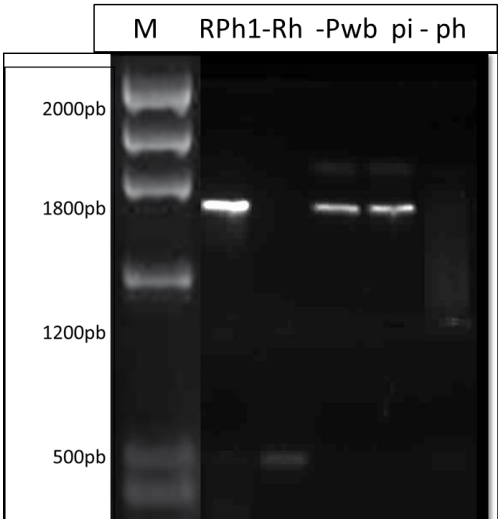

Fig. 3. PCR amplification of 16SrRNA(rDNA) sequence from various samples using primer pair P1 &P7, the PCR product (1.8kb) was separated by electrophoresis through 1% agarose gel. Lanes ((RPh1) rose phyllody, (pwb, pi) periwinkle witches'-broom inoculated by dodder) show PCR products of rose and periwinkle. Lanes: (Rh) rose healthy and (ph) periwinkle healthy. M: DNA marker, 100bp ladder (MoBiTec).

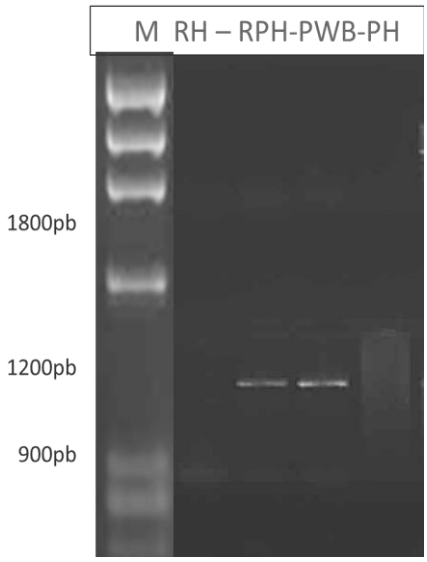

Fig. 4. Nested PCR amplification of 16S rRNA(rDNA) fragment(1.2kb) of phytoplasma genome using primers R16F2n/ R16R2 form PCR product amplified with primer P1 &P7. Lanes (RPH-PWB) show PCR products of rose phyllody and periwinkle witches'-broom plants representing symptoms. Lanes (RH, PH) represent rose and periwinkle healthy plants. M: pGEM DNA Markers (Promega).

These findings demonstrated and indicated the expected association of phytoplasma with diseased rose phyllody (RPh1) and periwinkle witches'-broom (PWB) symptoms.

Control of the diseases with tetracycline hydrochloride:

Two concentrations of tetracycline hydrochloride, *i.e.* 250 and 300 ppm, were used to control the phytoplasma diseases. Two different treatments were applied. Obtained results (Table 2) show that immersing transplants with tetracycline at 250 ppm for either 15 or 30 min gave 30 and 60% recovery in case of rose plants, 50 and 70% recovery in case of periwinkle plants. The percentage of recovery in case of rose and periwinkle at 300 ppm for either 15 or 30 min were 80 and 90%, respectively. On the other hand, treatment with 250ppm for 30min was better than that for 15min. Regarding the treatment with tetracycline as soil drench, it was observed that 250 and 300 ppm gave 30% and 60% recovery in rose plants, respectively, and gave30%and 80% recovery in periwinkle plants, respectively. So the recommended treatment is 300ppm as immersing transplants for 30min for both rose and periwinkle plants. In this treatment, the root uptake is effective and all the ingredients are translocated into the sites in which phytoplasma units are present.

Table 2. Tetracycline treatment of dodder inoculated rose and periwinkle plants

Treatment at tested concentration		Rose			Periwinkle		
		250 *	300	Control	250	300	Control
Soil drench		3/10**	6/10	0	3/10	8/10	0
Immersing transplants for (minutes)	15m	3/10	8/10	0	5/10	8/10	0
	30m	6/10	9/10	0	7/10	9/10	0

* Tetracycline concentration (ppm).

** No. of recovered plants /No. of treated ones (%).

D i s c u s s i o n

In the present study phytoplasma isolate was detected and characterized from naturally infected rose plants grown in different locations in Giza Governorate. The collected plants exhibited typical symptoms of phyllody. Amaral-Mello *et al*. (2006) and Khan *et al*. (2002) pointed out that phytoplasmas may alter the balance of hormones in the host plant, eventually inducing distortions of growth. They also stated that phytoplasmas produce certain proteins (*e.g*. glucanases and hemolysin-like proteins), which could act as virulence factors. In addition, phytoplasmas import numerous metabolites from the host plant, which eventually could change the physiological equilibrium of the host. On the other hand, Pracros *el al*. (2006) stated that expression of genes controlling the maintenance of the shoot apical meristem and the floral organ identity *ap3*, *ag*, *lfy*, were down regulated resulting in deformations and distortion of infected plants. On the other hand these symptoms are typical to those described by Xiaodong *et al*. (2009) and Jiménez and Montano (2010). Visual surveys of selected areas indicated an average of up to about 30% infection under field conditions. Rose plants used in this work exhibited symptoms very similar to rose phyllody symptoms reported in other countries (Anfoka *et al*., 2003).

The pathogenicity of the suspected phytoplasma was verified by both graft and dodder transmission. The obtained results indicated the pathogenicity of phytoplasma on the rose, as they were transmitted from diseased to healthy rose plants by both grafting and dodder. Moreover, the same symptoms were observed on tested plants .In almost all research work concerning phytoplasma diseases, grafting is the perfect method used for experimental infection with the tested phytoplasma (Al-zadjali *et al*., 2007).

Periwinkle plants were also experimentally infected with the types of phytoplasma by the same method and were kept as a maintaining host. Periwinkle plants are known to harbour almost all the known phytoplasmas, so it is used as an assay host for phytoplasma by grafting or dodder transmissions (Amaral-Mello *et al*., 2006 and Pracros *et al*., 2006). In the present investigation, the detected phytoplasmas were transmitted into periwinkle plants by dodder and used as a source of phytoplasma in PCR.

Examination of ultrathin sections of petioles tissues from leaves of periwinkle plants representing witches'-broom symptoms and rose plants with phyllody symptoms, revealed numerous phytoplasma units in the sieve elements. The morphology of the detected phytoplasma was the same in tissues of both periwinkle and rose. The morphology and structure of almost all the detected phytoplasma by electron microscopy of phytoplasma infected plant tissues indicated this aspect (Rita and Favali, 2003 and Xiaodong *et al*., 2009). The presence of phytoplasma units in phloem tissues of infected rose plants confirm the biological studies of graft and dodder transmission and the responsibility of the detected phytoplasmas for the diseases (EL-Banna *et al*., 2004).

The amplification of 16S rDNA of the phytoplasma in all symptomatic samples followed by preliminary grouping based on the sequences of the 16S rDNA gene was the first approach to classify this phytoplasma on a molecular basis (Schneider *et al*., 1999).

PCR amplification gave products of the expected molecular size (1.8kb) from phytoplasma-infected periwinkle and rose samples. The primer pair P1/P7 produced a PCR product from all phytoplasma groups tested, regardless of the plant host from which the DNA was extracted. According to the primer pair (P1/P7) adopted by many investigators (Salehi *et al*., 2006; EL-Banna *et al*., 2007and Ribeiro *et al*., 2007).

In the present study, nested-PCR assays with the primer pair R16F2n/R16R2 using the P1/P7 PCR amplified product as template (Chang, 2004 and Ribeiro *et al*., 2007) was applied. A product of approximately 1.2 kb DNA fragment, amplified from extracted DNA of rose and periwinkle plants representing phyllody, witches'-broom symptoms, respectively, was detected, it had not been possible to obtain phytoplasmal DNA that was sufficiently free of its respective host DNA. Therefore, the study of the phytoplasmal genome was hampered, and detection and characterization of phytoplasmal genes were performed randomly. Phytoplasmas were diagnosed almost solely on the basis of 16S rDNA sequences (Lee *et al*., 2000).

Results of the present study revealed that the rose phyllody in Egypt is associated with 16SrI group and this consequently contributes to demonstrate the diversity of phytoplasmas associated with this disease. A phytoplasma belonging to 16SrI, 16SrVI, 16SrV, 16SrXII and 16SrIII groups was also found in rose plants in USA, Jordan, Italy and Brazil, respectively (Anfoka *et al*., 2003 and Xiaodong *et al*., 2009).

Tetracycline hydroxide at 300 ppm for either 15 or 30 min gave 80 and 90% recovery in case of rose and periwinkle plants, respectively, by immersing transplants and at 300 ppm for each of rose and periwinkle plant for soil drench (60 and 80%, respectively). The inhibitory effect of tetracycline is attributed to that it contains deoxy-streptamine moiety which inhibits proteins of the microorganisms treated (EL-Banna *et al*., 2007 and Fucikovsky *et al*., 2011). Tetracycline and its derivatives are successfully used for controlling plant diseases caused by phytopathogenic Mallicutes.

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الكشف عن مرض الفيللودى المتسبب عن الفيتوبلازما في الورد ومكافحتة

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تم الكشف عن الفيتوبلازما المسببة لمرض phyllody من نباتات الورد المصابة اصابة طبيعية تم جمعها من مناطق مختلفه من محافظة الجيزة في مصر. تم اجراء اختبار النقل للفيتوبلازما المسببة لتلك الامراض من نباتات مصابة الي اخري سليمة باستخدام الحامول والتطعيم. وتم الكشف عن وحدات الفيتوبلازما في النباتات المصابة طبيعا وكذلك المنقول لها بالحامول والتطعيم في الورد والونكا بفحص انسجة اللحاء التي تم صبغها بصبغة داين بالمكرسكوب الضوئي.

استخدم الميكروسكوب الاكتروني النافذ للكشف عن وحدات الفيتوبلازما في انسجة لحاء نباتات الورد المصابة وكذلك انسجة الحامول التي استخدمت كنقطرة لنقل الفيتوبلازما وكان متوسط قطر تلك الوحدات يترواح مابين ٤٠٠ الي ٨٠٠ نانوميتر. تم استخلاص الحامض النووى DNA من النباتات المصابة والسليمة بطريقة CTAB. وتم استخدام Primer p1/p7 واعطي تقريباً ١٨٠٠ زوج من القواعد وتم استخدم بادئ متخصص R16F2n/R16R2 مع ناتج البادئ السابق وتم الحصول علي ناتج قدرة ١٢٠٠ زوج من القواعد.

تم استخدام تركيزان من التتراسيكلين ٢٥٠و٣٠٠ جزء في المليون بطريقتي غمر التربة لفترة اربع اسابيع متتالية وغمر الشتلات لمدة ١٥ و٣٠ دقيقة وكانت افضل النتائج باستخدام ٣٠٠ جزء في المليون لغمر التربة وغمر الشتلات لمدة ٣٠ في الورد و الونكا اعطي شفاء بنسبه ٩٠% للنبتات المصابة. وتعتبر هذه هى اول دراسة علي فيتوبلازما الورد تحت الظروف المصرية .