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Detection of Phytoplasma Associated with Periwinkle Virescence in Egypt

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Abstract: Little leaves, shortened internodes, virescence and witches' broom symptoms were observed affecting periwinkle plants growing in Kafr-el-sheikh governorate. These plants were examined for phytoplasma infections by electron microscopy. Phytoplasma appearing as spherical to ovoid structures of variable sizes were observed in phloem sieve tubes in diseased plants samples but not in symptomless plants samples by TEM. PCR-amplified phytoplasma of 16S rDNA using primers P1/P7 and PF2/PR2 was employed for the detection and identification of the phytoplasma associated with periwinkle plant. The Egyptian Phytoplasma Virescence (EPV) detected in diseased periwinkle (GenBank accession No. EF546439) was identified as members of aster yellow phytoplasma group (16SrI group) (*Candidatus* phytoplasma asteris). This is the first report on the occurrence of phytoplasma diseases in Egypt.

Key words: Periwinkle, virescence symptom, phytoplasma detection, electron microscope

INTRODUCTION

Phytoplasmas are non-helical wall-less prokaryotes, pleomorphic in shape that colonize the plant phloem were first reported almost 40 years ago and currently classified in the class Mollicutes (Firrao *et al*., 2005). Phytoplasmas infect numerous plant species including many ornamental plants (McCoy *et al*., 1989) inducing many symptoms including virescence, phyllody proliferation of shoots resulting in witches' broom, sterility of flowers, compact growth at the end of stems, yellowing, phloem necrosis and dieback of branches of woody plants (McCoy *et al*., 1989). Transmission Electron Microscope (TEM) allows the observation of characteristic phytoplasma morphology in sieve tubes of plant host (Doi *et al*., 1967; Sertkaya *et al*., 2005). The inability to isolate and culture phytoplasma *in vitro* has impeded their identification and classification. The introduction of PCR for assays in which universal primers derived from conserved 16S rRNA gene sequences are used has greatly improved the ability of researchers to accurately identify and classify a broad range of phytoplasmas (Ahrens and Seemüller, 1992; Firrao *et al*., 2005; Schneider *et al*., 1993; Vibio *et al*., 1994; Samuitienë and Navalinskienë, 2006). On the basis of the results of restriction fragment length polymorphism (RFLP) analyses of PCR-amplified 16S ribosomal DNA (rDNA), 15 distinct phytoplasma 16S rRNA groups and more than 38 subgroups have been identified (Lee *et al*., 1998; Marcone *et al*., 2000; Babaie *et al*., 2007). The phytoplasma agent has never been detected in Egypt, so the objective of this study was to determine association of phytoplasma with virescence disease in Periwinkle plant (*Catharanthus roseus*). The combined application of electron microscopy and molecular technique were used to verify phytoplasma presence and to identify them.

MATERIALS AND METHODS

Plant material: Leaves samples of periwinkle plants showing symptoms of virescence were collected from kafrelsheikh governorate, Egypt. Samples from symptomless plants were also collected. The study was carried out at Department of Plant pathology, Faculty of Agriculture, Kafrelsheikh university at the spring of 2007.

Electron microscope: Examination of ultra-thin section from symptomatic periwinkle leaves was performed according to the published procedure (Errampalli *et al*., 1991). The ultrathin sections were then examined using LEO912AB transmission electron microscope.

DNA extraction from plant: DNA was isolated using the procedure of Doyle and Doyle (1990). Approximately 1 g of tissue was frozen in liquid nitrogen and ground in a pre-chilled mortar and pestle. The powdered tissue was incubated in 10 mL of pre-heated extraction buffer (0.14 M sorbitol, 0.22 M Tris-HCl pH 8.0, 22 mM EDTA pH 8.0,

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0.8 M NaCl, 0.8% w/v CTAB, 1% N-lauryl sarcosine) and incubated for 20-30 min at 65°C with occasional shaking. We used the universal phytoplasma primers P1/P7 (Schneider *et al*., 1995), derived from highly conserved ribosomal sequences and priming the 5' end of the 16S rRNA gene and in the 5' region of the 23S rRNA gene, respectively, amplifying a 1853 bp fragment.

PCR amplification of 16S rDNA: The amplification of DNA was carried out according to Schneider *et al*. (1995). We used two universal primers P1 (5'AAG AGT TTG ATC CTG GCT CAG GAT T-3') and P7 (5'-CGT CCT TCA TCG GCT CTT-3') (Schneider *et al*., 1995) and two internal primers, PF2 (5'-GAG ATT CGC CAA AAA CTT GC-3') and PR2 (5'-GGT GCG TAG GCG GTT AAA TA-3'), which were designed in the 16S rDNA of the Egyptian periwinkle phytoplasma using Primer 3 program (http://seqtool.sdsc.edu/CGI/BW.cgi) and extended from position 567 to position 584 and from position 1247 to position 1266, respectively. The amplification run in a Peltier thermal cycler (PTC-200). The PCR products were electrophoresed on 1% agarose gels containing ethidium bromide and DNA bands were visualized using UV transilluminator.

DNA sequencing: PCR-amplified 16S rDNA from the Egyptian periwinkle phytoplasma was cloned and transformed into *Escherichia coli* using TOPO TA cloning Kit (Invitrogen) according to the manufacturer's instructions. White colonies were picked and transferred into Luria-Bertani (LB) medium containing 100 μg mL^{-1} ampicillin and incubated overnight at 37°C. The plasmid DNA was purified using the QIAprep Spin Miniprep Kit protocol (QAIGEN) and the purified plasmids were used for sequencing using automated DNA sequencing. The Egyptian periwinkle phytoplasma 16S rDNA was aligned with similar reference sequences of other phytoplasmas available in the GenBank nucleotide database using World Wide Web service ClustalW (www.ncbi.nlm.nih.gov) (Fig. 4). The alignments were used as input data to construct phylogenetic tree with the Neighbor-Joining method implemented in ClustalW. The tree was visualized with TreeView v. 1.6.1 program. The Full-length sequence of 16S rDNA of the Egyptian periwinkle virescence phytoplasma has been deposited in the EMBL/GenBank/DDBJ databases under the accession number EF546439.

RESULTS AND DISCUSSION

The symptoms of the diseased periwinkle plant included virescence and witches' broom and reduction in size of leaves.

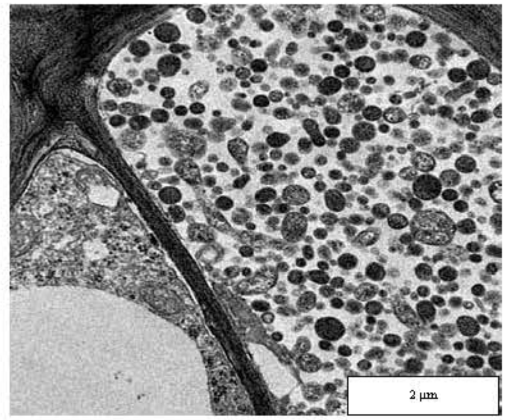

Fig. 1: Ultra-thin cross-section of the leaf midrib of periwinkle virescence phytoplasma plant showing cell filled with phytoplasma polymorphic bodies in phloem cells, (bar, 2 μm)

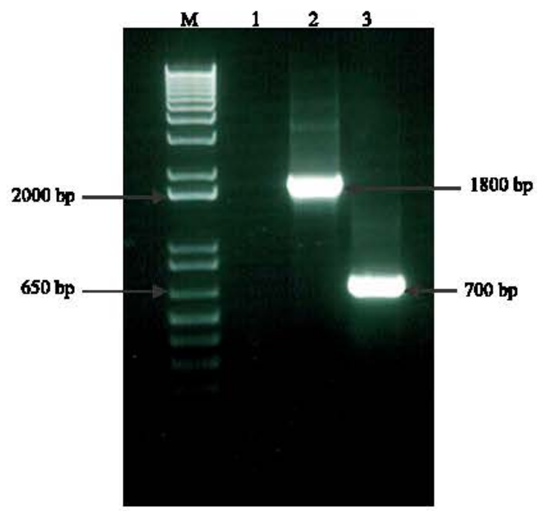

Fig. 2: Ethidium bromide-stained gel of the PCR amplification products of Periwinkle phytoplasma as obtained by using two primer sets. Lane 1 DNA from healthy periwinkle plant, Lane 2 amplified products by primer set P1-P7; Lane 3 amplified products by primer set PF2-PR2. Lane M is the 1-Kb plus DNA ladder (Invitrogen)

Electron microscope observation: The phytoplasma presence in the phloem sieve tubes was confirmed by examination of the ultra-thin cross-sections from periwinkle leaf midribs that showing symptoms of virescence and witches' broom that revealed considerable number of polymorphic bodies of mycoplasma-like organisms (MLOs) (Fig. 1). They were observed in mature

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        10        20        30        40        50        60        70
....|....|....|....|....|....|....|....|....|....|....|....|....|....|
TAAGAGTTTGATCCTGGCTCAGGATTAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAACGGAAGTTT
        80        90       100       110       120       130       140
....|....|....|....|....|....|....|....|....|....|....|....|....|....|
AAGCAATTAAACTTTAGTGGCGAACGGGTGAGTAACGCGTAAGCAATCTGCCCCTAAGACGAGGATAACA
       150       160       170       180       190       200       210
....|....|....|....|....|....|....|....|....|....|....|....|....|....|
GTTGGAAACGACTGCTAAGACTGGATAGGAGACAAGAGGGCATCTTCTTGTTTTTAAAAGACCTAGCAAT
       220       230       240       250       260       270       280
....|....|....|....|....|....|....|....|....|....|....|....|....|....|
AGGTATGCTTAGGGAGGAGCTTGCGTCACATTAGTTAGTTGGTGGGGTAAAGGCCTACCAAGACTATGAT
       290       300       310       320       330       340       350
....|....|....|....|....|....|....|....|....|....|....|....|....|....|
GTGTAGCCGGGCTGAGAGGTTGAACGGCCACATTGGGACTGAGACACGGCCCAAACTCCTACGGGAGGCA
       360       370       380       390       400       410       420
....|....|....|....|....|....|....|....|....|....|....|....|....|....|
GCAGTAGGGAATTTTCGGCAATGGAGGAAACTCTGACCGAGCAACGCCGCGTGAACGATGAAGTATTTCG
       430       440       450       460       470       480       490
....|....|....|....|....|....|....|....|....|....|....|....|....|....|
GTACGTAAAGTTCTTTTATTAGGGAAGAATAAATGATGGAAAAATCATTCTGACGGTACCTAATGAATAA
       500       510       520       530       540       550       560
....|....|....|....|....|....|....|....|....|....|....|....|....|....|
GCCCCGGCTAACTATGTGCCAGCAGCCGCGGTAATACATAGGGGGCAAGCGTTATCCGGAATTATTGGGC
       570       580       590       600       610       620       630
....|....|....|....|....|....|....|....|....|....|....|....|....|....|
GTAAAGGGTGCGTAGGCGGTTAAATAAGTTTATGGTCTAAGTGCAATGCTCAACATTGTGATGCTATAAA
       640       650       660       670       680       690       700
....|....|....|....|....|....|....|....|....|....|....|....|....|....|
AACTGTTTAGCTAGAGTAAGATAGAGGCAAGTGGAATTCCATGTGTAGTGGTAAAATGCGTAAATATATG
       710       720       730       740       750       760       770
....|....|....|....|....|....|....|....|....|....|....|....|....|....|
GAGGAACACCAGTAGCGAAGGCGGCTTGCTGGGTCTTTACTGACGCTGAGGCACGAAAGCGTGGGGAGCA
       780       790       800       810       820       830       840
....|....|....|....|....|....|....|....|....|....|....|....|....|....|
AACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTACTAAACGTTGGGTAAAACCAGTGTT
       850       860       870       880       890       900       910
....|....|....|....|....|....|....|....|....|....|....|....|....|....|
GAAGTTAACACATTAAGTACTCCGCCTGAGTAGTACGTACGCAAGTATGAAACTTAAAGGAATTGACGGG
       920       930       940       950       960       970       980
....|....|....|....|....|....|....|....|....|....|....|....|....|....|
ACTCCGCACAAGCGGTGGATCATGTTGTTTAATTCGAAGGTACCCGAAAAACCTCACCAGGTCTTGACAT
       990      1000      1010      1020      1030      1040      1050
....|....|....|....|....|....|....|....|....|....|....|....|....|....|
GCTTCTGCAAAGCTGTAGAAACACAGTGGAGGTTATCAGTTGCACAGGTGGTGCATGGTTGTCGTCAGCT
      1060      1070      1080      1090      1100      1110      1120
....|....|....|....|....|....|....|....|....|....|....|....|....|....|
CGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATTGTTAGTTACCAGCACGTAATG
      1130      1140      1150      1160      1170      1180      1190
....|....|....|....|....|....|....|....|....|....|....|....|....|....|
GTGGGGACTTTAGCAAGACTGCCAGTGATAAATTGGAGGAAGGTGGGGACGACGTCAAATCATCATGCCC
      1200      1210      1220      1230      1240      1250      1260
....|....|....|....|....|....|....|....|....|....|....|....|....|....|
CTTATGACCTGGGCTACAAACGTGATACAATGGCTGTTACAAAGGGTAGCTGAAGCGCAAGTTTTTGGCG
      1270      1280      1290      1300      1310      1320      1330
....|....|....|....|....|....|....|....|....|....|....|....|....|....|
AATCTCACGCAAGTTTTTGGCGAATCTCAAAAAAACAGTCTCAGTTCGGATTGAAGTCTGCAACTCGACT
      1340      1350      1360      1370      1380      1390      1400
....|....|....|....|....|....|....|....|....|....|....|....|....|....|
TCATGAAGTTGGAATCGCTAGTAATCGCGAATCAGCATGTCGCGGTGAATACGTTCTCGGGGTTTGTACA
      1410      1420      1430      1440      1450      1460      1470
....|....|....|....|....|....|....|....|....|....|....|....|....|....|
CACCGCCCGTCAAACCACGAAAGTTGGTAATACCCAAAGCCGGTGGCCTAACTTCGCAAGAAGAGGGAAC
      1480      1490      1500      1510      1520      1530      1540
....|....|....|....|....|....|....|....|....|....|....|....|....|....|
CGTCTAAGGTAGGGTCGATGATTGGGGTTAAGTCGTAACAAGGTATCCCTACCGGAAGGTGGGGATGGAT
      1550      1560      1570      1580      1590      1600      1610
....|....|....|....|....|....|....|....|....|....|....|....|....|....|
CACCTCCTTTCTAAGGAAACAATTATCATCTTCAGTTTTGAGAGACTTAAGAAAGTTTTTCATTGTAACT
      1620      1630      1640      1650      1660      1670      1680
....|....|....|....|....|....|....|....|....|....|....|....|....|....|
TGCTTGCAAATTGTATTTGCAACATTTTAATCTTTTTAAGATTAAGGGCCTATAGCTCAGTTGGTTAGAG
      1690      1700      1710      1720      1730      1740      1750
....|....|....|....|....|....|....|....|....|....|....|....|....|....|
CACACGCCTGATAAGCGTGAGGTCGGTGGTTCAAGTCCATTTAGGCCCACCATAACCACAAATAGGCAAA
      1760      1770      1780      1790      1800      1810      1820
....|....|....|....|....|....|....|....|....|....|....|....|....|....|
ATCTTAAAAAAGCTCTTTGAAAAGTAGATAAACGAAGGTTAAAAAATCAAAGGAACTAAGGGCGCACAGT
      1830      1840      1850
....|....|....|....|....|....|...
GGATGCCTTGGCACTAAGAGCCGATGAAGGACG
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Fig. 3: Nucleotide sequence of 16S rDNA gene complete sequence; 16S-23S rDNA intergenic spacer, complete sequence and 23S rDNA gene, partial of Egyptian periwinkle virescence phytoplasma (EPV), EMBL/GenBank/DDBJ accession number EF546439

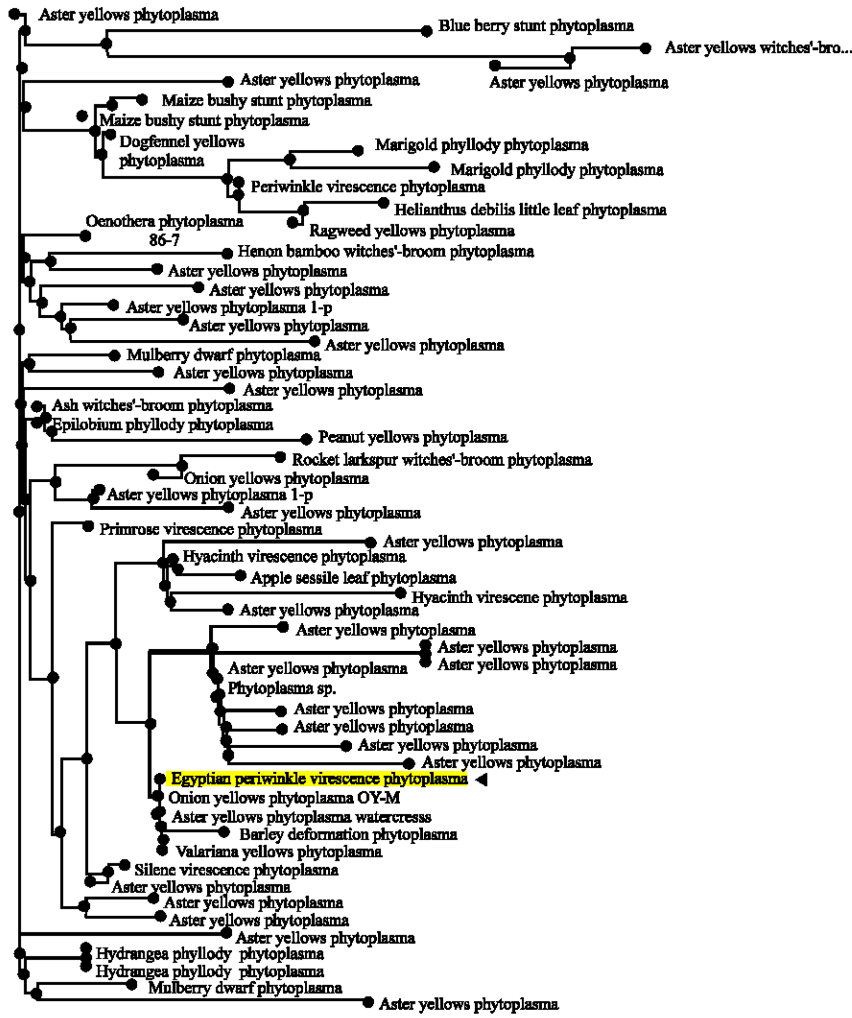

Fig. 4: Position of Egyptian Periwinkle Virescence phytoplasma (EPV) in a phylogram of 16S rDNA sequences generated using the Neighbor-Joining method implemented in ClustalW. The tree was visualized with TreeView v. 1.6.1 program

and immature phloem sieve tubes. Cells filled by the micro-organisms were also seen. The majority of particles were ovoid or spherical with size, some were irregular in form or elongated. However, no MOLs were detected in the phloem tissues of symptomless plants. Transmission Electron Microscopy (TEM) has traditionally been used to demonstrate the presence of phytoplasmas in phloem tissues (Chang *et al*., 1996; Hwang *et al*., 1997). The morphological characteristics of the structures are similar to organisms found in plants infected with yellows-type diseases (Doi *et al*., 1967; Bertaccini *et al*., 1999; Sertkaya *et al*., 2005). The presence of phytoplasmas in ultrathin sections of diseased periwinkle and the absence of other pathogens in the symptomless ones support a phytoplasma aetiology of the disease.

Amplification, cloning and sequencing of periwinkle phytoplasma 16SrDNA: Amplification with universal primers, P1/P7 and the designed internal primers, PF2/PR2 of infected periwinkle plant, after 35 cycles, the amplification product was the expected size of about 1,800 and 700 bp, respectively (Fig. 2). The DNA bands were typical for phytoplasmas, when the universal primer pairs are used to amplify 16S rDNA (Schneider *et al*., 1995). No amplification was observed when DNA from healthy periwinkle plant was used in place of DNA template in the reaction mixture (Fig. 2). Sequence alignment using BLAST (www.ncbi.nlm.nih.gov/BLAST//blast.cgi) suggested that the full sequence of 16S rDNA of (EPV) is approximately 1540 (Fig. 3).

A phylogenetic tree was constructed after the periwinkle phytoplasma 16S rDNA sequence was compared with the sequences of other MLOs in GenBank database (Fig. 4.). This tree shows that the Egyptian Periwinkle Phytoplasma (EPV) clusters with the other

MLOs which we studied and is closely related to Barely deformation phytoplasma, Onion yellows phytoplasma, Aster yellows phytoplasma watercress and valeriana yellows phytoplasma, which are belonging to the aster yellow phytoplasma group (16SrI group) (*Candidatus* phytoplasma asteris) with identity 98%. The phylogenetic position of EPV is shown in Fig. 4. Phytoplasma was named Egyptian periwinkle virescence phytoplasma (EPV). Sequence analysis of 16S rDNA was used to characterize phytoplasmas in more details and to determine their phylogenetic relationships to each other (Namba *et al*., 1993; Seemüller *et al*., 1994). Moreover, the availability of sequences from 16S rDNA has made the rDNA a prime target for phytoplasma detection by PCR (Seemüller *et al*., 1994). Genetically different phytoplasmas have been identified in diseased ornamentals. However, they are mainly members of the 16SrI group, subgroups B, C and A (d'Aquilio *et al*., 2002; Kaminska *et al*., 2003; Rojas-Martinez *et al*., 2003; Bertaccini *et al*., 2005; Siddique, 2005). This is in agreement with present data showing the phytoplasma infecting periwinkle plant in Egypt plants is assigned to (*Candidatus* phytoplasma asteris).

The distribution and transmission characteristics of phytoplasmas are still unknown in the country. Detailed investigations are necessary to determine many important aspects of the epidemiology of the diseases caused by phytoplasmas in Egypt.

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