Detection and molecular characterization of phytoplasma associated with Hibiscus Witches'-Broom in Egypt

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Abstract
Hibiscusrosa-sinensisis a valuable ornamental species widely planted in Egypt. The Phytoplasma associated witches'-Broom has been detected on symptomatic hibiscus plants. Samples of hibiscusleaves were collected from El Giza, Alexandria, Qlubia,El-Fayom, and El-Mansouragovernoratesand analyzed for phytoplasma infection.The collected hibiscus plants showed characteristic symptoms for Phytoplasma associated witches'-Broom disease, which is characterized by excessive axillary branching, abnormally small leaves, and deformed flowers. Dienes' stain was used for detection of witches' broom infection midribs of the symptomatichibiscus plant. The phloem of infected tissues showed scattered area stained bright blue. Molecular detection utilizing nested and direct PCR as well as DNA sequencing was used for the diagnosis of the witches' Broom infection. Total DNA was isolated from leaf tissues of infected hibiscus plants. Nestedpolymerase chain reaction(PCR) was performed using the universal - phytoplasma specific primers; P1/P7, R16F2n/R16R2.Witches' broom specific primers SR1/ SR2, at the spacer region (SR), were used for the direct PCR. Amplicons of expected size characteristic for phytoplasma associated witches' broom were obtained from infected hibiscus samples. DNA sequencing and phylogenetic analysis of the 16S rRNA gene fragment from the hibiscus witches' broom phytoplasma showed 100% homology with the same region of thehibiscus witches' broom strain thatisolatedin Brazil (PhytoplasmaBrasiliense). The DNA sequence was submitted for the gene bank as the first report of the Brasiliensephytoplasma associated witches' broom affecting hibiscus in Egypt.

Key words: Hibiscus, Dienes' stain, 16SRNA, phytoplasma, PCR, Sequencing.

Introduction
Red Hibiscus (rosa-sinensis) is a widely grown evergreen ornamental herbs, shrubs and trees of thetropics and sub-tropics which are grown as landscape plants, attractive roadside plants, border plants or as acontainer plants in greenhouses (Houet al; 2005). It's a valuable species widely planted in Egypt. The Red Hibiscus belongs to thefamilyMalvaceae and are reported to possess various medicinal propertieslike, regulation of menstrual cycle, curing hypoglycemia, potentiate hair growth,anti-hypertensive, anti-tumor, antioxidant and even treatment of venerealdiseases (Telefoet al.,1998; Herrera, 2004;Chang et al .,2006and Temitope, 2010).

The 16SrII group of the Witches'-broom phytoplasma diseaseis a group of severe plant disorders caused by obligate, cell wall-less bacteria, which are responsible for important yield losses in many crops worldwide (Lee , et al., 2000 and Bertaccini, 2007) including ornamental plants and fruit crops. Infected plants show a wide range of symptoms. The

phytoplasma associated witches' broom disease was characterized by leaf yellowing and malformation, as well as by short internodes (Hogenhoutet al., 2008; Arismendiet al., 2010; Bertaccini and Duduk, 2009).

Phytoplasmas associated with the witches' broom, of the 16SrII group, have been recorded worldwideon different crops and become a problem in hibiscus culture as well. It have been found in the many plants in the Middle East, Mediterranean region, Australia, Mexico, Israel, Indonesia, Egypt, and Chile (Bertaccini and Duduk, 2009; Khan et al., 2007; and Arismendi et al., 2010). This disease has become a major problem in Hibiscus culture in Braziland in Australia(Helena et al 2001).

Light microscope staining methods have been described as a quick, simple method for detecting phytoplasma diseases (Deeley et al 1979). Those involved often specialized fluorescent microscopy (Hibbenet al 1986 and Fránováet al., 2007).The first approaches were mainly based on the observation of symptoms caused by different strains and the observation of the phytoplasma presence in sections of phloem tissues when Dienes' stained (Musetti, 2013). However, many issues in phytoplasma detection can occur making it difficult to have an accurate phytoplasma diagnosis. They can include an unbalanced distribution of the phytoplasma in plant organs as well as the recovery phenomenon and a low concentration (especially in field-collected samples where the amount of phytoplasma DNA is less than 1% of the total amount of plant DNA) (Bertaccini, 2009 andFirrao et al., 2007).

Although microscopic techniques are still important for detecting phytoplasmas (Chapman et al., 2001; Fránováet al., 2007), PCR based assays using universal (generic) or broad-spectrum primers designed based on conserved sequences of phytoplasma DNA (e.g. 16S rRNA, 16S-23S spacer region, and DNA-fragments sequences) allow the detection of a wide array of unknown phytoplasmas associated with different plant diseases (Sinclair and Griffiths , 2000). Nested PCR assays were designed to increase both sensitivity and specificity of detection of phytoplasma diseases (Lee et al., 2000). Universal primers, but specific for phytoplasmas, based on the regions of the 16S rRNA gene, intergenic region, and 23S rRNA gene are used (Smart et al., 1996;Heinrich et al., 2001).Furthermore, sequencing of PCR products helps in the specific identification and characterization of phytoplasma groups (Lee at al., 1998; 2000). However, the majorities of phytoplasma reports in Egypt are based on electron microscopy of ultrathin section, light microscopy, graft and dodder transmission, and DNA amplification with PCR using universal primers (Ammaret al., 2005;Om-Hashem, 2007); Only recent work has involved PCR-using specific primers and DNA sequencing for a more specific identification of these prokaryotes.

The objectives of this study were to detect and characterize the phytoplasma in tissues of diseased hibiscus plants using Dains' stain light microscopy and molecular based techniques. Molecular characterizationwas performed using the DNA sequencing and phylogenetic analysis of the spacer region between 16S and 23S rRNA fragment of the isolated phytoplasma genome. This work concerning phytoplasma associated witches' broom (group 16SrII) diseases of hibiscus plants is achieved for the first time in Egypt.

Materials and methods:

Plant samples and symptoms of phytoplasma.

Symptomatic leaves of hibiscusrosa-sinensiswere collected from five differentlocations in Egypt; El- Giza, Alexandria, Qlubia,El-Fayom, and El-Mansoura. Witches' broom disease was observedon hibiscus plants showing the identical symptoms of phytoplasma infection.

Light microscopy of Dienes'staind tissues:

Dienes' stain was used to detect witches' broom disease caused by phytoplasmainhibiscus plants. The used Dienes' staincontained 2.5g Methylene blue, 10g Maltose,1.25g Azure II and 0.25g Sodium carbonate dissolved in 100ml Water.The stain was filtered through filter paper Whatman No1.Free hand section, of infected leaves midrib of Hibiscus rosa-sinensis were prepared. The prepared sections were transferred onto 70% ethanol then stained using Dienes' stain for 10 min, and washed with distilled water. After washing, the section was examined by light microscope at x 330 times (Hibbenet al 1986 andMusetti 2013).

Nucleic acid extraction and PCR:

The total DNA was extracted from hibiscus plant tissues using plant DNA extraction kit (sigma, USA). The DNA extracted from symptomatic and/or asymptomatic hibiscus plants was used as template for PCR. The Universal phytoplasma-specific primers,P1/P7 and R16F2n/R16R2 were used to amplify the 16SrRNA and 16S/23, spacer region of the phytoplasma genome in nested PCR(Leeet al., 2004, and Bhatet al., 2006). The primer pair P1/P7 was used - in the first step PCR- for the amplification of 1.8 kb product of 16S rRNA gene. 1 µl DNA extracted from hibiscus plants was used in 25µl total PCR mixture contained 25 pmol of each primer; 200 µM of each dNTP; 1x polymerase reaction buffer; 2.5 mM MgCl2; 1.25 U of dream-Taq polymerase (Fermentas) and sterile water to a final volume of 25 µl. 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 72C°.

The primer pair R16F2n, R16R2 used to amplify a 1.2 kb fragment of 16S rRNA gene in the second step nested-PCR as described by (Wang and Hiruki 2001). One µl of DNA amplified by direct PCR with primer pair P1/P7 from hibiscus samples were used at 1:10 dilution as template for nested-PCR. The nested PCR 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 for 2 min at 50°C, and primer extension at 72°C for 3 min. A final extension step was added for 10 min 72C°.

Hibiscus witches' broom was detected through direct PCR using witches' broom-specific primers SR1: AGGCGGATC CTTGGGGTTAAGTCGTAA and SR2: AGGCGAATTCCGTCCTTCATCGGCT CTT representing the phytoplasma-specific 16S/23S rRNA (rDNA) intergenic spacer region (SR) (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 15 s at 94 °C, 15 s at 55 °C and 30 s at 72 °C, with a final extension of 10 min at 72°C. All PCR products were stained with gel star (Lonza, USA) and separated on 1% agarose gel with 1xTBE buffer then analyzed using (Gel Doc 2000 Bio.RAD). The molecular weight of the PCR products were determined by comparison with 100 bp and/or 1 kb DNA ladder.

DNA Sequencing:

The 1.2 kb fragment of 16S rRNA gene in the second step nested-PCR was purified and directly sequenced using Automated DNA sequencing. The forward and the reverse primers R16F2n/R16R2 were used for DNA sequencing. The nucleotide sequence was analyzed using DNAMAN Sequence Analysis Software (LynnonBioSoft. Quebec, Canada) and compared with those of phytoplasma strains available in GenBank.

Results:

Symptoms:

Symptoms of hibiscus witches' broom disease caused by phytoplasma were observed on hibiscusornamental plant growing in El-, Giza, Alexandria, Qlubia,El-Fayom, and El-Mansoura governorates. The infectedplants were characterized with the following symptoms, excessive axillary branching, leaf yellowing, short internodes, proliferation of shoots, abnormally small leaves, deformed flowers, and in some cases, premature flower dropping.Highlyinfectedplants may die due to severe infection (Fig.1).

Light microscopy of Dienes' stained tissues:

The previously stained hand sections of hibiscus leaves midrib were examined using light microscope.Fig.2 shows that, phloem was colored bright turquoise blue indicating the presence of phytoplasma in midrib sections prepared from infectedhibiscus sample. The phloem of sections prepared from healthy samples remained unstained

Nested PCR:

The universal-phytoplasma specific primer pairs; P1/P7 and R16F2n / R16R2 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 analysis. 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. Healthy plants used as negative controls didn't show any PCR amplified products (Fig. 3 A). Electrophoresis analysis for the PCR products showed clear bands at the expected size, only when the template DNA was extracted from phytoplasma-infected hibiscus samples.

Witches' broom-specific PCR detection:

Samples showed positive results for the nested PCR, using the primer pairs R16F2n/R16R2,were analyzed for hibiscus witches' broom. The direct PCR for detection of the hibiscus witches' broom was performed using the specific primer pair SR1/SR2 and yielded a strong band of approximately 325 bp for DNA extracted from hibiscus samples showed witches'-broom symptoms, (Fig. 3 B). No bands were detected for healthy plant controls. These findings demonstrated the

expected associations of a phytoplasmawith diseased hibiscus witches broom exhibiting reduction in size leaf proliferation of lateral shoots, and stunting symptoms.

Sequence analysis

Nucleotide sequencing of the 1246 kb purified PCR fragment of 16S rRNA gene was performed and compared with sequences of other phytoplasma strains available in GenBank. The obtained data were analyzed using DNAMAN software. Multiple sequence alignment of thenucleotide sequence of our PCR fragment was done with the corresponding sequences of the otherphytoplasma strains on GeneBank (Fig. 4). Nucleotide sequence for our findings was submitted to the gene bank and accessioned by GenBank accession number "**KF716175**"

Fig. (1):Symptoms of the phytoplasma associatedwitches' broom on Hibiscus plants. A: Healthy Hibiscus plant control. B, C and D: infected plants showing leaf yellowing, short internodes, proliferation of shoots, and premature flower dropping.

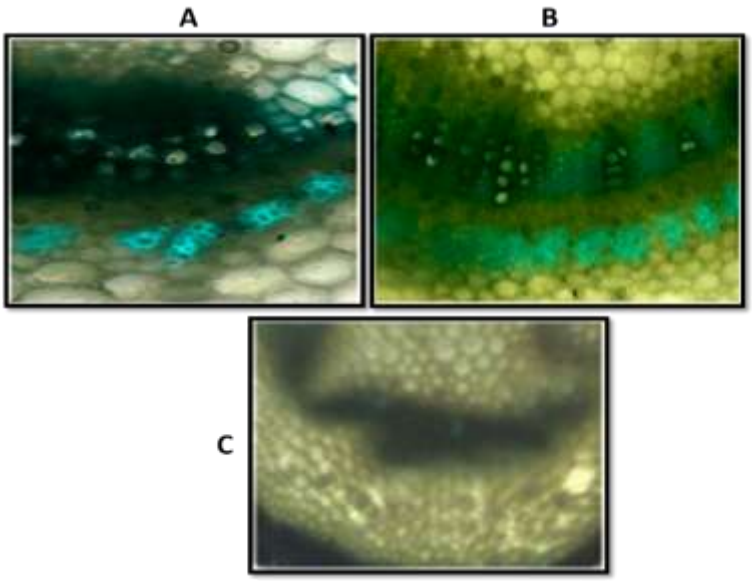
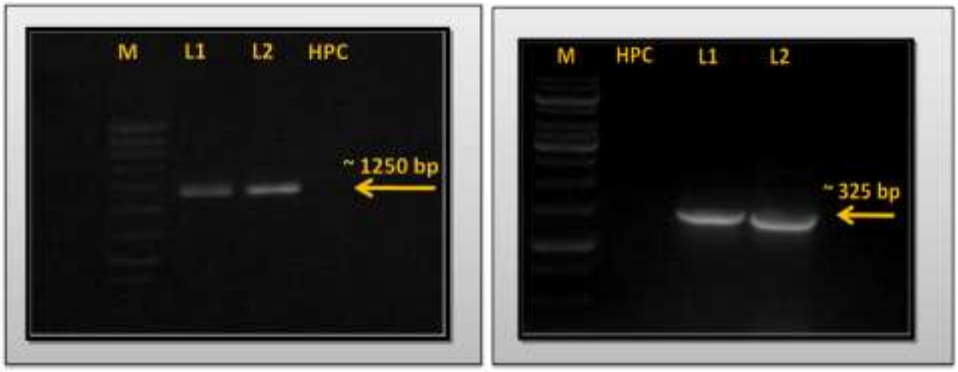


Fig. (2): Cross section of leaf midrib from Hibiscus witches' broom, showing phloem tissue stained with dark blue color after treatment with Dienes'stain,A and B compared with the healthy plant control; C at magnification of 330 X.



[A] **[B]**

Fig. 3: Gel electrophoresis for the detection of the phytoplasma in Hibiscus usingUniversal phytoplasma-specific [R16] PCR primers. M: 1 Kb DNA Ladder. L1 and L2 are different samples showed W. B symptoms. HPC: Healthy Plant Control [A]; and using witches' broom-specific PCR primers. M: 100 bp-plus, DNA Ladder. L1 and L2 are different samples showed W. B symptoms. HPC: Healthy Plant Control [B].

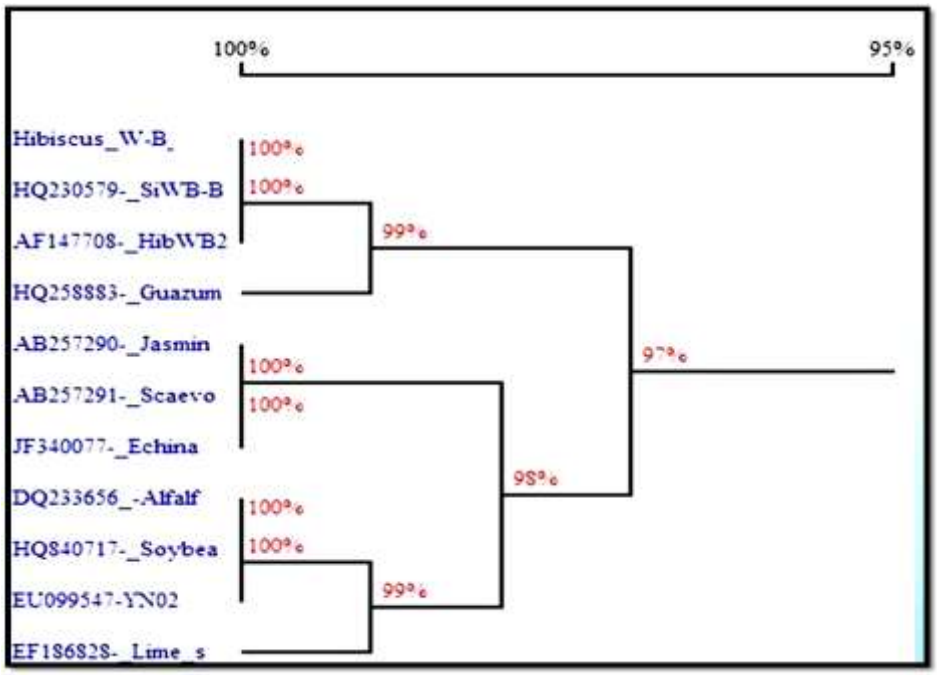


Fig 4:Sequence analysis and the phylogenetic tree showed range of 99-100%. Similarity with the different phytoplasma strains in the GeneBank. Our isolate is indicated in the tree as "Hibiscus_W.B_".

Discussion:

Phytoplasma is considered as one of the most important plant pathogens reducing the productivity of several economic crops including hibiscus. Sensitive detection and characterization as well as the diagnosis of phytoplasmasare of paramount importance for effective prevention strategies, particularly because phytoplasmas may have avery long latency period.

We detected the presence of a phytoplasma in association with hibiscus using both classical and molecular tools. The main light microscopy was used successfully for the diagnosis, as a preliminary method, to assess the presence of phytoplasmas in the infected hibiscus tissues which agreed with Musetti, 2013.

Molecular diagnosis confirmed the infection of hibiscus plants with Phytoplasma Associated with Witches'-Broom based on the symptoms associated with phytoplasma diseases. Suspected plants were sampled for the detection and molecular characterization of the phytoplasma.A nested-PCR approach was followed for the detection of the phytoplasma in suspectedleaf samples as mentioned in materials and methods section.

PCR amplifications were obtained by direct, as well as nested PCR for the DNA extracted from Hibiscus tissue. The nested PCR using the universal phytoplasma- specific primers P1/P7 and R16F2/R16R2 gave amplification of the expectedfragment at1.2 kb, while the direct PCR using the witches' broom-specific PCR primers (SR1/SR2) showed

a clear band at ~ 325 bpcorresponding to the 5'-end of the 23S rDNA and the partial 16S rRNA gene. The first round of the nested PCR using the P1/P7 primers didn't show a band due to the low phytoplasmatic DNA concentrations as compared to plant DNA. That agrees with the fact that, the detection of phytoplasmatic DNA was achieved only with a nested-PCR (Hamedet al., 2013). In the second PCR reaction, conditions are optimized and the sensitivity of the technique is increased facilitating visualization of the ampliconsthat refers to the use of the PCRproduct of the first Round PCR as a DNA template for the second one. DNA amplified from templateDNA isolated from any of the healthy, non-symptomatic, plant samples werefound to be negative for phytoplasma presence in both direct and nested PCR.Those PCR results clearly demonstrated the natural infection of hibiscus with phytoplasma associated with Witches'-Broom.

Phylogenetic analysis and homology with the sequence of the 16S ribosomal RNA (rRNA) gene, the 16-23S rRNA spacer region, and the 5`-end of the 23S rRNA gene identified the phytoplasma as belonging to the 16Sr II group (97-99% homology).

Sequence obtained from the1246 bp PCR product associated with infected Hibiscus rosa-sinensis was submitted to BLAST analysis which showed a 100% similarity with reference strain of hibiscus witches' broom from Brazil, belonging to 16SrII-group.

DNA sequencing and phylogenetic analysis indicated that this phytoplasma clustered in the 16SrII group. A 1,246 bp sequence of the 16S rRNA gene from the hibiscus witches' broom phytoplasma showed 100% homology with the 16S rRNA gene, hibiscus witches' broom in Brazil (HQ230579-SiWB-B) and peanut witches'-broom (EU099547-YN02).

Nucleotide sequence determined in this study was submitted to the gene bank and accessioned by Gen Bank accession number "KF716175" as the first report of phytoplasma infection affecting hibiscus witches' broom in Egypt.

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