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RESEARCH

Detection and molecular identification of phytoplasma associated with Gazania in Egypt

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ABSTRACT

Background: Phytoplasmas a wall-less microorganism belongs to Class Mollicutes cause diseases in several commercial ornamental plants, leads to serious economic losses all over the world. During 2017-2018, gazania showing phyllody, yellowing, proliferation, virescence and little leaf symptoms was observed Giza, Egypt.
Objective: The current work aims the detection and molecular identification of phytoplasma infecting Gazania in Egypt.
Methods: Phytoplasma disease was detected and isolated from naturally infected gazania plants during surveys in flower nurseries and open filed in Giza governorate, it was transmitted from naturally infected Gazania to healthy periwinkle and other ornamental plants by dodder (*Cuscuta reflexa*), and the leaf hopper (*Empoasca decipiens*). Free hand sections stained with Diene's stain was used to detect and differentiate the phloem tissues of leaf sections from infected gazania and periwinkle plants. Transmission Electron Microscopy (TEM) revealed the presence of phytoplasma in the sieve tubes and parenchyma cells of leaf midribs in infected plants. DNA extracted from symptomatic samples was used as a template in nested polymerase chain reaction (nested-PCR) using universal primer pairs P1/P7 and R16F2n/R16R2. Sequencing and phylogenetic analysis were performed to identify the detected phytoplasmas.
Results: Phytoplasma was transmitted successfully from naturally infected gazania to healthy ornamental plants by dodder and insect. Transmission electron microscopy (TEM) revealed that, phytoplasma-like bodies were detected inside phloem, sieve tubes and parenchyma cells of leaf midribs tissues in infected plants and ranging from 200 to 400nm in diameters. The 16SrRNA gene from phytoplasma was amplified by nested-PCR assay and direct sequenced using specific primer pairs. Phylogenetic tree was based on obtained sequences data.
Conclusion: The phytoplasma associated with gazania exhibiting phyllody, yellowing, proliferation, virescence and little leaf symptoms was confirmed by the results of LM and TEM observations and nested-PCR testing. Based on direct sequence date, phylogeny analysis, the associated phytoplasma was classified as related to 16SrII group.

Keywords: Phytoplasma; Gazania; Light Microscopy; Electron microscopy; nested-PCR.

BACKGROUND

Gazania (*Gazania rigens*) plants are member of the daisy family *Asteraceae*, genus Gazania. Also known as the treasure flower or the African daisy and grown for decorative purposes in gardens and landscape design projects, as a houseplant. Phytoplasmas, cell wall-less phytopathogenic bacteria, belonging to the class Mollicutes prokaryotes that colonize plant phloem and insects, which are associated with numerous devastating diseases in ornamental plants worldwide. Epidemics of these diseases have forced the withdrawal of many ornamental plant varieties from cultivation (Bertaccini, 2007; Chaturvedi *et al*., 2010; Hogenhout *et al*., 2008; Lee *et al*., 2000; Maejima *et al*., 2014). Symptoms are generally phyllody, yellowing, proliferation, virescence, little leaf, stunting of plants and reduced size of flowers are commonly observed on ornamental plants and their occurrence often results in high economic losses.

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(Bellardi *et al.*, 2018; Bertaccini *et al.*, 2014; Chaturvedi *et al.*, 2010; Ermacora and Osler, 2019) .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, as well as the methods of transmission by dodder, and insect transmission (Akhtar *et al.*, 2009; Franova *et al*., 2007; Galetto *et al*., 2011; Marcone *et al.*, 1997).

Diene's stain was used to detect plant diseases induced by phytoplasma like organisms (Deeley *et al*., 1979; Musetti and Favali, 2004). Light microscopy and transmission electron microscopy of plants exhibiting characteristic phytoplasma symptoms were carried out by many investigators. (Devonshire, 2013; El-Banna *et al.*, 2015; Musetti, 2013; Nejat and Vadamalai, 2013; Omar *et al.*, 2008). DNA extracted using as a template in nested polymerase chain reaction (PCR) using universal primers pairs P1/P7 and R16F2n/R16R2 or DNA sequencing and phylogenetic analysis allow to detection and diagnosis of a wide array of unknown phytoplasmas associated with plants (El-Banna *et al*., 2015; El-Sisi *et al*., 2017; ElSayed and Boulila, 2014; Singh *et al*., 2011; Youssef *et al.*, 2017). According to the available literature no previous reports were published on the occurrence of phytoplasma in gazania plants in Egypt before the present work was carried out. The goals of this study were detection and molecular identification of phytoplasma associated with gazania in Egypt.

MATERIALS AND METHODS

Source of samples:

During 2017-2018, samples were collected from infected commercial flower nurseries, botanical gardens and different open filed in Giza governorate showing the identical symptoms of phytoplasma infection. All the infected plants kept under greenhouse conditions (25-30°C) and used as a source of infection and some studies in the following experiments.

Transmission studies and Etiology

Dodder transmission

Seeds of dodder (*Cuscuta reflexa*) were surface sterilized with 1% sodium hypochlorite solution, washed with distilled water then *in vitro* germinated on petri-dishes 12cm diameter bottomed with wetted filter paper for 12days at room temperature (22-25°C), sown in plastic 20cm pots containing a 1:1 mixture of vermiculite and peatmoss. According to the method of Mikhail *et al.*, (2012); Moustafa (1990), germinated dodder seeds (10 seeds/infected plant) were used as bridge to transmit gazania associated phytoplasma onto healthy gazania and periwinkle (*Catharanthus roseus*) plants and kept in an insect-free cage till symptoms development.

Insect transmission

Leafhopper (*Empoasca decipiens*) colonies were collected from gazania fields during August and September, Adults leafhopper individuals were allowed to oviposit on periwinkle plants in insect-proof cages, and hatching nymphs were transferred weekly onto healthy periwinkle plants in cylindrical cages (Fig. 2). These steps were repeated twice till insect matured into adults, insect randomly collected from these colonies always tested negative in PCR analysis, as described by Borth *et al*., (2006). After starving for 24h, the phytoplasma free insects were allowed to feed on infected gazania plants for 1week as acquisition period. About 10 and 20 insects/plant were placed on healthy gazania and periwinkle plants for inoculation access period of 30 days and the plants were then sprayed to kill adult insects and monitored till symptoms appearance.

Preparation of free hand sections for Diene's stain

Diene's stain was used as a preliminary method to detect phytoplasmas in infected plant tissues. Samples were taken from stems and petioles of naturally, artificially infected and healthy plants. Free hand cross sections were prepared and transferred to Diene's stain for 5 min as described by Deeley *et al.,* (1979); Musetti (2013); Musetti and Favali (2004) .The stained sections were later washed in distilled water and examined immediately by light microscope. (LEICA ICC50 HD).

Anatomical studies (light and electron microscopy)

This work was carried out in Fac. Agric. Res. Park (FARP), transmission Electric Microscope (TEM) Lab. Faculty of Agriculture Cairo University. The material examined included samples of leaf petioles of symptomatic gazania plant. Tissues pieces of about 2×2 mm samples were prepared according to the method descripted by El-Banna *et al.,* (2007); Rocchetta *et al.,* (2007).

Light microscope (semi thin sections)

Stems and petioles from infected and healthy plants were sectioned approximately (500-900 nm thick) with ultra-microtome (Leica model EM-UC6). For light microscopy samples representing leaf petioles were specimens killed and fixed for at least 48hrs in F.A.A. (10ml formalin, 5 ml glacial acetic acid and 85 ml ethyl alcohol 70%) the selected materials were washed in 50% ethyl alcohol, dehydrated in a normal butyl alcohol series, embedded in paraffin wax of melting point 56˚C, sectioned to a thickness of 20 micron, double stained with safranin and fast green, cleared in xylene and mounted in Canada balsam (Nassar and El-Sahhar, 1998).

Electron microscopy (ultrathin sections)

For electron microscopy samples were prepared and sectioned (90 nm thick) with the ultra-microtome, mounted on copper grids (400 mish). Sections were stained with uranyl acetate and lead citrate, and allowed to air dry as descripted by El-Banna *et al.,* (2007); Musetti and Favali (2004). Stained sections were examined by transmission electron JEOL (JEM-14l00) at the candidate magnification. Images were captured by CCD camera model AMT. (ZeissEM9.60KVFacarZeiss.Oberkochen).

Molecular biology studies

DNA isolation and detection procedures

The total DNA was isolated from 1g of fresh samples of infected and healthy gazania leaves exhibiting phyllody symptoms, periwinkle plants and leafhoppers insect as well, using Dellaporta extraction method (Dellaporta *et al*., 1983). Nucleic acid was precipitated by adding 2.5 volumes absolute ethanol and were collected by centrifugation, washed in 70% ethanol, dried and re-suspended in 50 µL TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0). DNA was used immediately or stored at -20˚C before use in nested-PCR.

PCR based assay:

Direct PCR assays were performed with primer pair P1/P7 (Schneider *et al*., 1995). 1 µL of extracted DNA was used in 25 µL total PCR reaction mixture contained 25 pmol of each primer; 12.5 µL Mangotaq DNA polymerase (Bioline GmbH, Luckenwalde). The product of the direct reaction was diluted 1:10 with nuclease free water (Promega, USA), and used as template

for nested PCR with primers R16F2n/R16R2 (Deng and Hiruki, 1991). The DNA amplification was started with a denaturation step at 94°C for 3 min followed by 35 cycles consisting of denaturation at 94°C for 30 Sec., annealing at 53°C for 1min extension at 72°C for 2 min, a final extension step was added for 7 min at 72°C. The PCR products were stained with EZ View nucleic acid stain (Biomatik, USA) and analyzed by electrophoresis in 1.0% agarose gel and visualized by UV illumination.

Nucleotide sequencing

The PCR product of the spacer region R16F2n/R16R2 of the phytoplasma genome was gel extracted using Gel/PCR DNA Fragments Extraction Kit (Geneaid, Taiwan). The purified PCR product was directly sequenced using automated DNA sequencing in both directions. The nucleotide sequences were assembled and analyzed using DNAMAN sequence analysis software (Lynnon Corporation, Canada) ver.7, the nucleotide sequences were compared with the corresponding sequences of nineteen sequences of other phytoplasma isolates from different geographical regions that were deposited in the GenBank.

RESULTS

Symptomatology and Transmission

Identical phyllody disease symptoms associated with phytoplasma infection were observed on gazania plants included yellowing, proliferation, virescence and little leaf symptoms and stunting of plants and reduced size of flowers were naturally observed on ornamental plants from commercial flower nurseries, botanical gardens and different open filed in Giza governorate (Fig. 1).

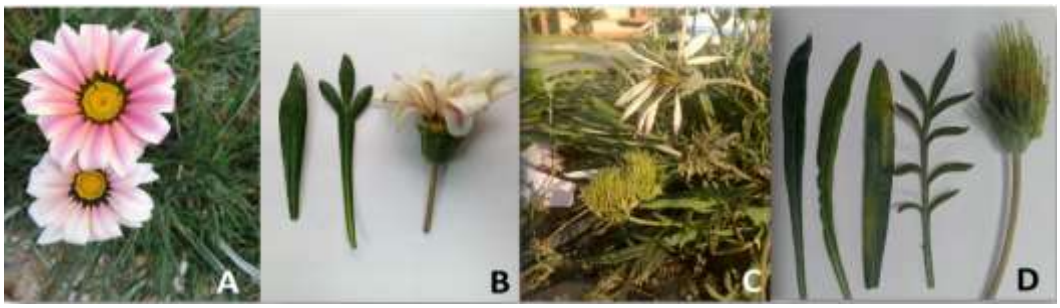

Fig. 1: Symptoms. A and B: Healthy gazania plant control. C and D: infected plants showing phyllody, yellowing, proliferation, virescence and little leaf symptoms observed on naturally infected gazania plants.

Dodder plant was used to transmit gazania associated phytoplasma onto healthy gazania and periwinkle plants. Results showed that the phyllody disease was transmit successfully from infected to healthy plants and symptoms were observed 4weeks post transmission and results were confirmed with nested-PCR (Fig. 2).

Fig. 2: Dodder transmission: A) in vitro dodder germination on petri-dishes (B, C) symptoms of phyllody disease symptoms on gazania and periwinkle plants by dodder transmission.

Insect transmission

The leafhopper (*Empoasca decipiens*) individuals were collected from gazania fields during August and September with phytoplasma, phyllody disease was transmitted successfully and was variable when assayed with transmission experiments. About 50% of healthy gazania and periwinkle plants became infected with phytoplasma when high populations of leafhopper of (20 insects/plant) were used in transmission tests. However, about 15% were become infected when groups of (10 insects/plant) were caged on individual test plants (Fig. 3).

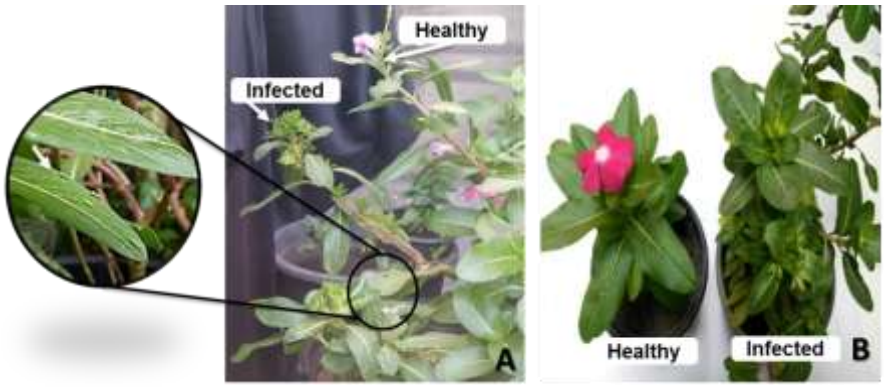

Fig. 3: Insect transmission: (A) the transmission trials with, *Empoasca decipiens* (B) Symptoms on periwinkle plants by insect transmission

Examination of free hand sections stained with Diene's stain

By using Diene's stain infected and healthy gazania leaf petioles were identified. The diseased phloem area was always being stained dark blue (Fig. 4), while the phloem of sections prepared from healthy plants remained unstained.

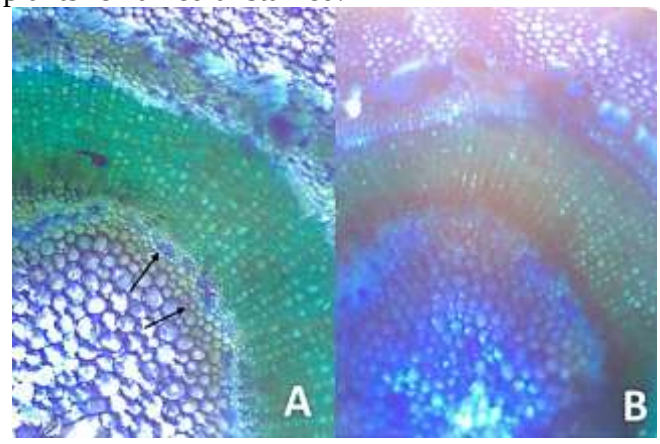

Fig. 4: Micrograph of transverse section of infected and healthy gazania plant petioles stained with Diene's stain. (A) The blue areas in phloem cells indicating phytoplasma presence and (B) Cross section of healthy petioles of gazania plant.

Anatomical studies (light and electron microscopy)

Light microscope (semi thin sections)

Light microscopy of semi thin sections approximately (500-900nm thick) with ultra-microtome was carried out from healthy and infected leaf petioles of symptomatic gazania plant. Investigation of representing sections of petiole tissues showed several anatomical changes. These anatomical changes were more pronounced in the bundle area including the phloem units (Fig. 5).

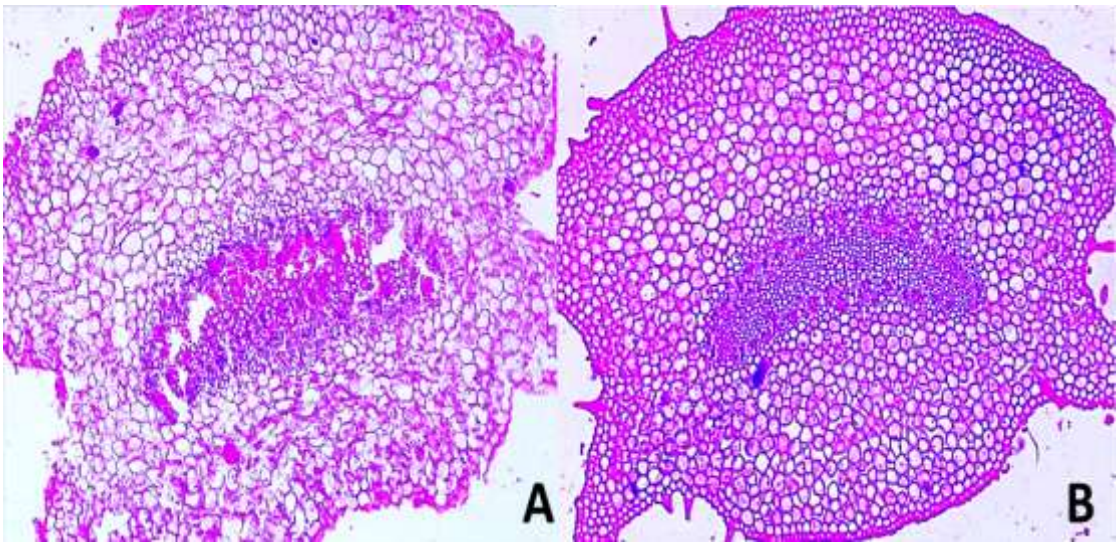

Fig. 5: (A) Cross sections in petioles of phyllody infected gazania plants. (B) Cross section in petioles of healthy gazania plants, X= 10.

Electron microscopy of ultrathin section:

The presence of phytoplasma in the phloem tissue of petioles of infected gazania and periwinkle were investigated through the ultra-thin section examination by transmission electron microscope and the examination revealed the presence of numerous phytoplasma units in the sieve elements of the infected plants arranged next to the cell membrane these units were rounded, elongated or pleomorphic bounded by a unit membrane, lacking cell wall and measuring 200-400 nm in size and no phytoplasma particles were found in healthy samples (Fig. 6-A, B).

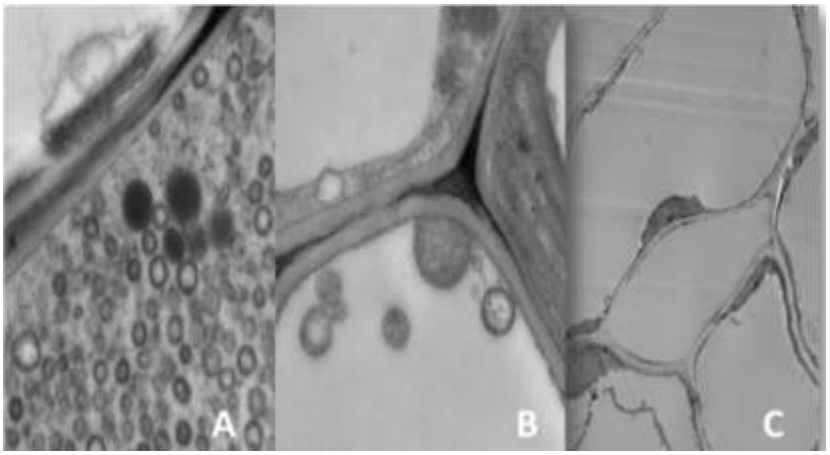

Fig. 6: Electron micrograph representing: (A) cross section of sieve tubes containing phytoplasmas unites, (B) Showing phytolasma units as rounded elongated, measuring 200-400 nm in size and in budding. Stage x= 25000. (c) Cross section of sieves tubes healthy.

Molecular biology studies

Molecular characterization of phytoplasma

Total DNA was isolated successfully from fresh samples of infected and healthy gazania, periwinkle plants and the leafhoppers and used as a template for direct PCR using the universal primers pair P1/P7 and nested PCR amplification using R16F2n/R16R2, respectively, all symptomatic samples generated amplicons of about 1200bp when used as a template as a result for the nested PCR amplification. No visible bands were detected from the corresponding healthy samples (Fig. 7).

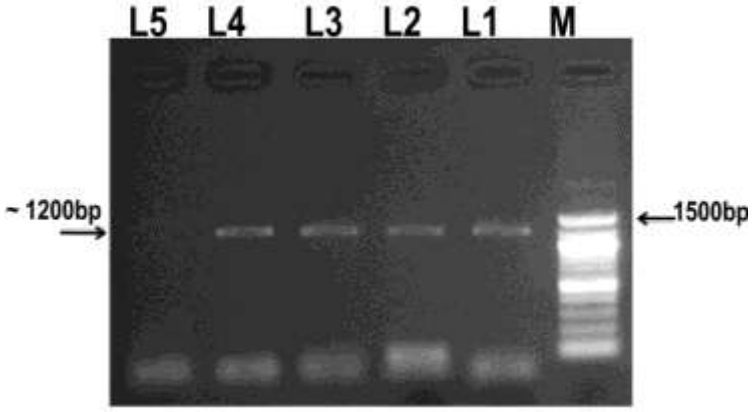

Fig. 7: Agarose gel electrophoresis of PCR products from 16S rDNA gene using universal phytoplasma specific primers R16F2n/R16R2 of phytoplasma in symptomatic gazania, periwinkle plants and leafhoppers insect. L1: naturally infected gazania plant showed phyllody symptoms, L2 and L3: periwinkle plants previously inoculated with phytoplasma using dodder and leafhoppers L4: leafhoppers insect, L5: Healthy gazania plant and M: 100bp DNA Ladder.

Sequence analysis

The Purified PCR product**s** were directly sequenced in both directions and compared with the corresponding sequences of other isolate of phytoplasma available in GenBank, the obtained data were assembled with DNAMAN sequence analysis software. The nucleotide sequence was submitted to the GenBank with accession number "MK377249.1" as Gazania-EGY strain.

Multiple sequence alignment and phylogenetic analysis was conducted to compare the Egyptian strain (Gazania-EGY) with the corresponding sequences of other phytoplasma isolates from different geographical regions available in GenBank (Fig. 8). Results showed that Gazania-EGY isolate was 99.4% and 98.7% similar to Sesame phyllody phytoplasma [16SrII] (Acc. No. EU072505) and candidates *Phytoplasma aurantifolia* [16SrII] (Acc. No. U15442.1) from Iran and Oman respectively. The sequence result also observed that the identity was about 96.9% with Hibiscus witches'-broom phytoplasma [16SrXV] (Acc. No. AF147708.1) from USA and 91.5% and 91.3% identity with Weeping tea witches'-broom (Acc. No. AF521672.1) and Awka wilt phytoplasma [16SrXXII-A] (Acc. No. Y14175.1) from Australia and UK respectively, sequence analysis observed that the isolate under study is belonging to 16SrII group.

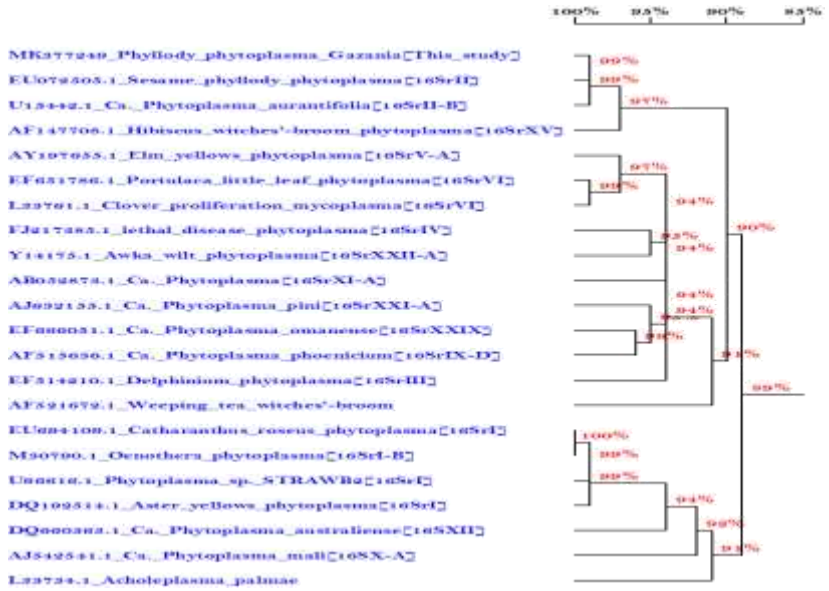

Fig. 8: Phylogenetic tree constructed by DNAMAN ver.7, showing the phylogenetic relationships for the partial 16SrDNA nucleotide sequences for Egyptian isolate (Gazania EGY) phytoplasma sample, compared with 19 representatives from other 16S rDNA groups available in GenBank.

DISCUSSION

Phytoplasma is considered as one of the most important plant pathogens reducing the productivity of several economic crops and ornamental plants including gazania plants (Bertaccini, 2007; Lee *et al*., 2000). In the current study, phytoplasma was detected and characterized from naturally infected gazania grown in different locations in flower nurseries and open filed in Giza governorate. The collected samples exhibited typical symptoms of phyllody, yellowing, proliferation, virescence and little leaf symptoms. These symptoms are characteristic to phytoplasma infection and are typical. These symptoms are similar to those described by Bellardi *et al.*, (2018); Chaturvedi *et al.*, (2010); Ermacora and Osler (2019). Transmission of phytoplasma from naturally infected to healthy gazania and periwinkle plants using the parasitic

plant dodder (*Cuscuta reflexa*) is an effective way to maintain and propagate the phytoplasma under study. At the same time, the healthy dodder is one of the main ways by which phytoplasma infection is achieved under artificial conditions (Marcone *et al*., 1997; Moustafa, 1990). The same symptoms that are typical for the phyllody disease post inoculation. This result agreed with similar to previous studies on phytoplasma transmission by the dodder (Akhtar *et al.,* 2009; Hamed *et al.,* 2014; Salehi *et al.,* 2009). In Egypt, this insect (*Empoasca decipiens*) has a very broad range of host plants, which makes them efficient vectors of pathogens residing in those host plants. Accordingly, the existence of these insects in gazania flower nurseries and cannot exclude mainly attracted to the phloem sieve tubes of infected plants then function as a vector therefore, be taken as an indication for the dispersion of phytoplasma infection in gazania flower nurseries which also corresponded with experimental transmission results as evident from observed symptoms such as, phyllody. This result agreed with similar to previous studies on phytoplasma transmission by insect (Galetto *et al*., 2011; Mahmoud *et al*., 2011).

Light microscopy techniques have been used successfully as preliminary methods for diagnosis to verity the presence of phytoplasma in symptomatic plants so they constitute the first steps towards understanding the possible association between phytoplasma and the disease symptoms in the plants. Infected samples stained with Diene's, showed that some phloem cells are stained with blue color which indicating the presence of phytoplasma compared with healthy plants these results is similar to those described by Deeley *et al.,* (1979); Hamed *et al.,* (2014); Musetti (2013); Musetti and Favali (2004). Light microscope (LM) (semi thin sections) and transmission electron microscopy (TEM) were used to studies the anatomical changes in infected plants and to detect phytoplasma units inside the infected tissues of infected plants has traditionally been used to demonstrate the presence of phytoplasmas in phloem tissues (Hwang *et al.,* 1997). Phytoplasmas were demonstrated in high numbers in sieve elements of inoculated plants, and were usually appear as rounded or irregular bodies, with a diameter of 200-400 nm, the reliability of phytoplasma diagnosis in phloem tissue by TEM technique, was reported and discussed by Franova *et al.,* (2007); IRPCM and Spiroplasma (2004); Musetti and Favali (2004); Omar *et al.,* (2008) in molecular diagnosis confirmed the infection of Gazania plants with phytoplasma associated with phyllody disease for the first time in Egypt. The DNA extracted from gazania and periwinkle plants and, those showed phyllody symptoms, to detect phytoplasmas in DNA samples nearly using Nested-PCR is performing by preliminary amplification using a universal primers pair followed by second amplification using a second universal primer pair. By using a universal primer pair followed by PCR using a group specific primer pair, nested PCR is capable of detection of multiple phytoplasmas present in the infect universal phytoplasma primers designed on sequences of the 16S-23S rRNA spacer region (SR) (Smart *et al.,* 1996; Smart *et al.,* 1996).Those PCR results clearly demonstrated the natural infection of gazania and periwinkle plants with phytoplasma associated with phyllody disease and confirmed the successful transmission of the phytoplasma associated with phyllody disease into the healthy gazania and periwinkle plants using dodders and insects .

CONCLUSION

In the present study, phytoplasma was detected and characterized from naturally infected gazania grown in different locations in flower nurseries and open filed in Giza governorate. The collected samples exhibited typical symptoms of phyllody, yellowing, proliferation, virescence and little leaf symptoms. These symptoms are characteristic to phytoplasma infection. The pathogenicity of the suspected phytoplasma was verified by insect and dodder transmission. The

obtained results indicated that phytoplasma in gazania which were transmitted to periwinkle exhibited the same symptoms. Examination of ultrathin sections from healthy and infected plants diseased revealed the presence of numerous phytoplasma units in sieve tubes of phloem leaves but not in healthy plants, the anatomical changes observed during the present investigation were indicated through visualizations of semi thin sections. For molecular detection and characterization of phytoplasma in gazania and periwinkle plants. Nested PCR was also used for detection of phytoplasma in the samples collected from the citrus and periwinkle plants. The two universal primer pair's p1 p7 and R16F2n/ R16R2 designed for amplification of phytoplasma 16S rDNA were used to detect phytoplasma.

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The authors declare that they have no competing interests.

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