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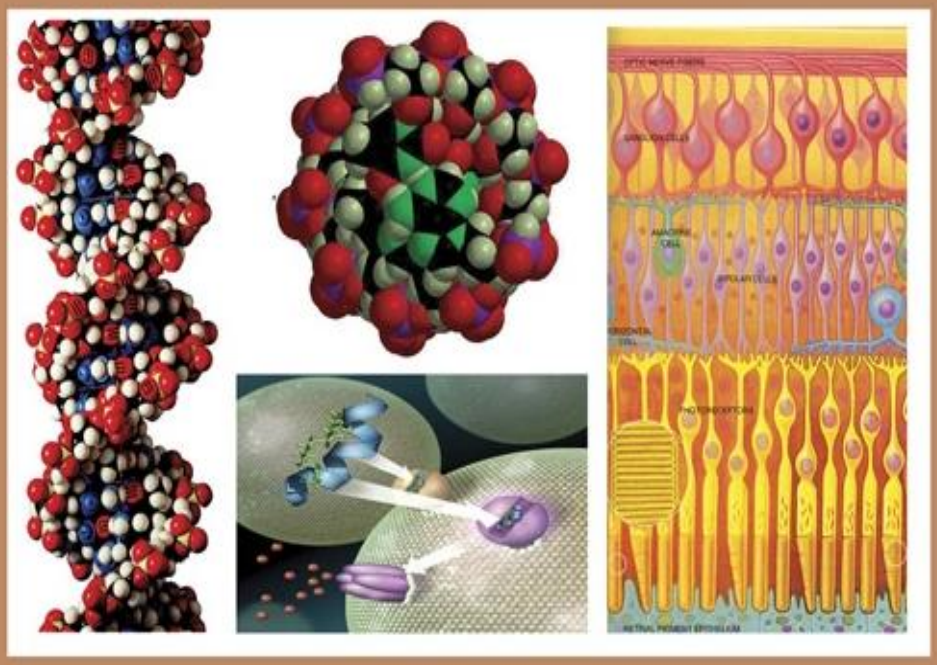

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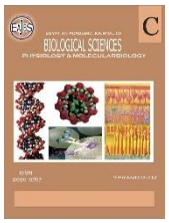

First report of *Candidatus* Phytoplasma Asteris Infecting Lily in Egypt

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ABSTRACT

During the inspection of imported grown Lily (*Lilium* spp.) plants (Acapulco cultivar), symptoms of *Candidatus* Phytoplasma asteris (*Ca*. P. astris) infestation was observed in the experimental fields of the Faculty of Agriculture, Cairo University, Giza governorate. The observed disease symptoms included stunting, scorched and leaf discoloration, flowering bud failure, and reduced quality of bulbs. Nested polymerase chain reaction (PCR) was the key technique employed in this study. Four pairs of primers were used in PCR analysis. In the first amplification, a universal primer pair P1/P7 was used to amplify a 1.8 kbp DNA fragment of the rRNA operon. In the second nested amplification, the universal primer pairs R16F2n/R16R2 (F2n/R2) and R16F0/R16R1 (F0/R1) and the specific P1/Ayint were used to detect 1.2, 1.44, and 1.5 kbp, respectively, from aster yellows group phytoplasma infecting plants. Containment of phytoplasma infestation in lily was obtained through a periodical spray of infected lily plants with tetracycline hydrochloride antibiotic. Lily plants are vegetatively propagated. This allows the spread of this phytoplasma to several economic crops.

INTRODUCTION

Phytoplasmas are small, cell wall-less bacterial pathogens of plants that colonize plant phloem and insects (Lee *et al.*, 2000). Because of the lack of cell walls, they are polymorphic with variable sizes from 0.2 to 0.8 µm. Phytoplasmas are transmitted by leafhoppers, *Cicadelloidea,* and planthoppers, *Fulgoroidea,* vectors. Phytoplasmas can also be spread by vegetative propagation, grafting, cuttings and through plant parasitic species. In nature, Phytoplasmas have been associated with hundreds of plant diseases affecting vegetables, cereal and oilseed crops, fruit crops, trees, ornamentals, and weeds (Lee *et al.*, 2000; McCoy *et al.,* 1989; Bertaccini et al., 2005, 2014). Phytoplasma incidence in economic crops, trees, oil seeds, and ornamental crops in the Middle was comprehensively reviewed by Hemmati *et al.* (2021).

Aster yellows (AY) group (16SrI) phytoplasmas are associated with more than 100 economically important diseases worldwide and represent the highly diverse and widespread phytoplasma group (Lee *et al.,* 2004). *Ca.* P. astris is a novel phytoplasma taxon associated with aster yellows and related diseases (lee *et al.,* 2003, 2004). Phytoplasmas infecting lilies was reported in Poland and the Czech Republic (Bertaccini *et al.,* 2002, 2005). In Egypt, phytoplasma of the 16SrI was characterized in *Dodonaea viscosa* (Mokbel, 2020). Another phytoplasma, belonging to the 16SrII-D subgroup was also detected in periwinkle (*Catharanthus roseus*) in Egypt (El-Sisi *et al.,* 2017).

During the inspection of lily (*Lilium* sp.), [Acapulco cultivar], plantation, grown, under covers, from imported bulbs, in the experimental greenhouses, at the Faculty of Agriculture, Cairo University Giza governorate, symptoms mimic those caused by Ca. P. astris infestation was observed. These included stunting, scorched and leaf discoloration, flowering-bud failure, and malformed and necrotic bulbs.

The objective of this study is to illustrate the presence of a newly introduced phytoplasma species in lily plants for the first time in Egypt which might represent a threat to locally grown lilies and other flowers and economic crops due to the wide host range of this phytoplasma. Further, this study is concerned with demonstrating the effect of tetracycline hydrochloride antibiotics in controlling *Ca.* P. astris. Tetracyclines were effective in reducing the accumulation of *Ca*. P. asteris and other phytoplasmas (Ishiie *et al.,* 1967; McCoy *et al.,* 1982; Kaewmanee *et al.,* 2011).

MATERIALS AND METHODS

Source of Infected Plants:

Lily bulbs were imported from Jordan and planted at the greenhouse facilities of the Horticulture Department, Faculty of Agriculture, Cairo University, Giza, Egypt. Emerging leaves showing phytoplasma symptoms were used as plant sources for the detection of phytoplasma.

DNA Isolation and Primers for Polymerase Chain Reactions (PCR):

The total nucleic acid of plant samples was extracted from leaf tissue using the procedure described by Doyl and Doyl (1987). Nucleic acids (from both diseased and healthy tissues) were diluted with sterile bi-distilled water to a final concentration equal to 20 ng/µl. Nested PCR was the key technique employed in this study. Four pairs of primers (Fig.1 & Table 1) were used in PCR analysis. In the first amplification, a universal primer pair P1/P7 was used to amplify a DNA fragment of about 1.8 kbp that extends from the 5' end of the 16S rRNA gene to the 23S rRNA gene. In the second nested/hemi nested amplification, the universal primer pairs F2n/R2 and F0/R1 and the specific P1/AYint (for AY group) were performed to detect 1.2, 1.44, and 1.5 kbp respectively, from AY group phytoplasma-infecting plants.

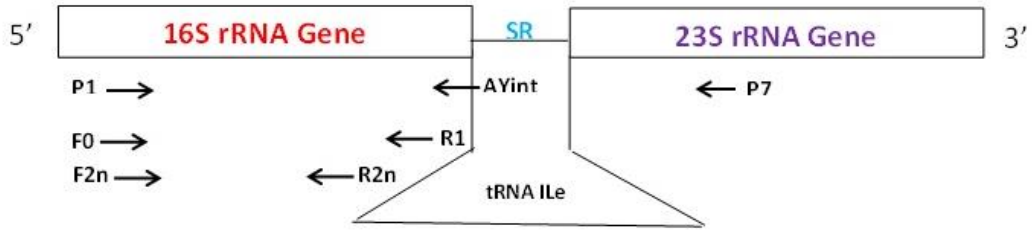

Fig. 1. A cartoon indicating the phytoplasma rRNA operon including the 16S, the 23S rRNA genes and the intergenic separating region (SR). The positions of oligonucleotide primers used in PCR analysis were represented by arrows. The drawing is modified from Smart *et al.*, 1996).

Table 1: Description of oligonucleotide primers used for PCR amplification

Primers used	Oligonucleotide Sequences (5'….3')	Location*	Product	References
P1	AAG AGT TTG ATC CTG GCT CAG GAT T	16S-23S rRNA genes,	*Ca* 1800 bp	Deng & Hiruki, 1991; Smart et al., 1996
P7	CGT CCT TCA TCG GCT CTT			
R16F2n (F2n)	CAT GCA AGT CGA ACG GA	16S rRNA genes,	*Ca* 1200 bp	Lee et al., 1993
R16R2 (R2)	TGA CGG GCG GTG TGT ACA AAC CCC G			
R16F0 (F0)	CTG GCT CAG GAT TAA CGC TGG CGG C	16S rRNA genes;	*Ca* 1440 bp	Davis and Lee, 1993
R16R1 (R1)	TTC CCT CTT CTT GCG AAG TTA GGC CAC CGG			
AYint	TAC AAT TTG CAA AGG CAT TAC	16S-Separating region (SR)	*Ca* 1500 bp	Smart et al.,1996

*** Locations of primers within the rRNA, 16SrRNA gene, 23SrRNA gene, or the separating region, SR**

PCR assays were performed in a final volume of 25 µl/0.2 ml PCR tube containing: 20 ng DNA templet (lee *et al.*, 1993), 2.5 mM MgCl2, 5X Go Taq DNA polymerase reaction buffer (Cat No. M8301, Promega Madison, WI, USA), 0.2 mM dNTPs, 10 mM of each primer, and 1.25 U of Taq polymerase. PCR experiments were conducted in an automated Techne TC-3000G Thermal Cycler. The following PCR conditions were used: An initial denaturation (94°C for 3 min) was followed by 35 cycles of each: denaturation (94°C for 1 min), annealing 50°C for 2 min, and extension (72°C for 3 min). A final extension cycle (72°C for 10 min) was followed. In each run, a negative control, i.e., PCR mix devoid of DNA template, was included. PCR products were examined by electrophoresis through 1% agarose gel, prepared in TAE buffer, followed by staining in ethidium bromide (0.5µg/ml) and visualization with a UV transilluminator. Usually in nested/hemi nested PCR, the DNA products resulting from the P1/P7-primer runs were diluted (0 to 50) times with water.

Chemical Control of Phytoplasma Infecting Lily:

Six weekly consecutive spray applications of 250 mg/L (=250 ppm) of water-soluble tetracycline hydrochloride, (Chemical Industries Development, Giza, Egypt) were used to spray 10 lily plants developing phytoplasma syndrome and grown under greenhouse conditions. Two weeks after the last application of the antibiotic, three-leaf samples from each plant were collectively macerated and exposed to DNA extraction and PCR analysis using P1/P7 and R16F2n / R16R2 primers.

RESULTS

Symptoms of Phytoplasma on Lily Plants:

The observed disease symptoms included leaf epinasty and discoloration, leaf malformation, leaf and bud scorching, stunting, and, flowering-bud failure (Fig. 2). Infected lily bulbs showed declined and necrotic roots compared to healthy bulbs (Fig. 3).

Detection of *Ca.* P. Astris in Lily with PCR Using Universal and Specific Primers:

Total DNA extracts from infected lily plants were successfully purified with an average $A_{260/280}$ ratio of 2.1 (data not shown). In nested and hemi nested PCR, the universal primer pairs F0/R1 (Fig. 4) and the specific P1/Ayint primer pairs (Fig. 5) used the 1,8 kb amplicons, amplified by P1/P7 primers, to respectively amplify 1.44, and 1.5 kbp of the DNA templets. The primer pairs F2n/R2 detected phytoplasma presence in the tissues of the lily (Fig. 6).

Effect of Tetracycline in Controlling Phytoplasma in Lily Plants:

Infected lily plants sprayed with 250 ppm for 6 consecutive weeks showed symptom recovery from phytoplasma (Fig. 6). Upon testing these plants with PCR using the universal primer pairs F2n/R2, only

seven plants out of ten were phytoplasma free while three plants, though symptomless, were positive for phytoplasma presence (Fig. 7).

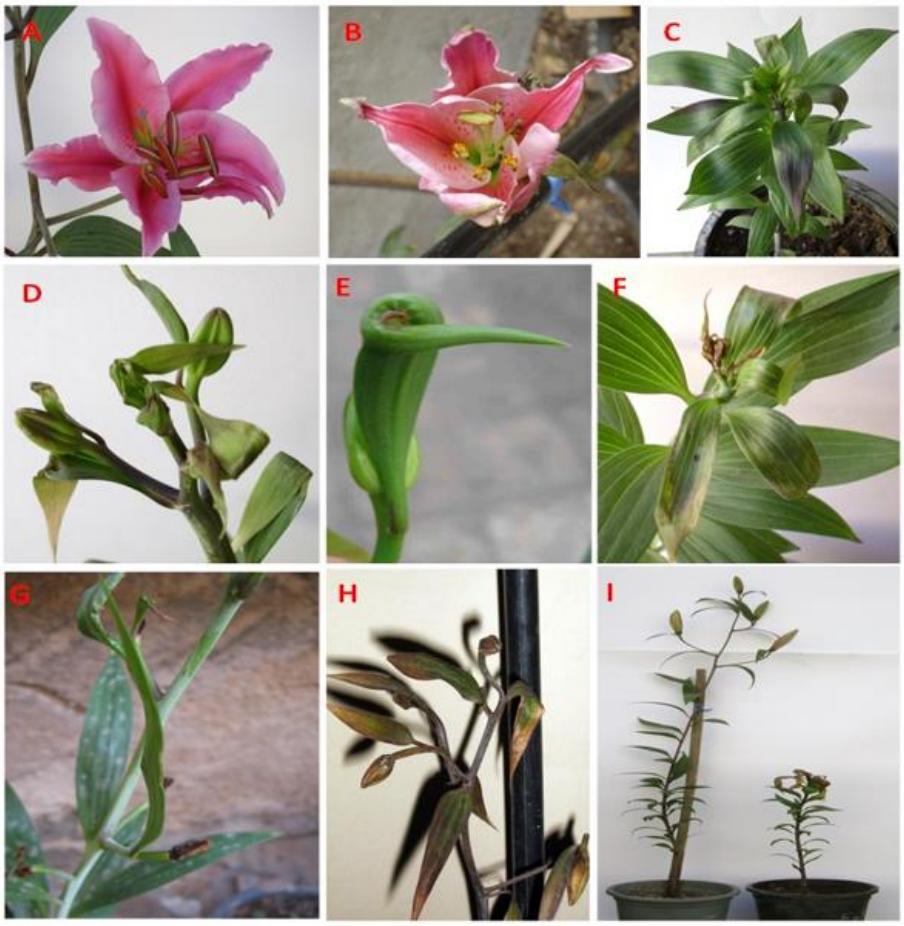

Fig. 2. Developing of phytoplasma- disease symptoms on lily plants. **A,** healthy flower; **B**, diseased flower showing flower-deterioration parts; **C,** leaf epinasty and purple top symptoms; **D,** leaf malformation; **E,** leaf twisting; **F,** leaf scorching; **G,** bud scorching; **H,** complete leaf and bud scorching; **I,** stunting symptoms (right plant) comparing to the healthy plant (left).

Fig. 3. Symptoms of *Ca.* P. asteris infection on lily bulbs (right) comparing to healthy bulbs (Left)

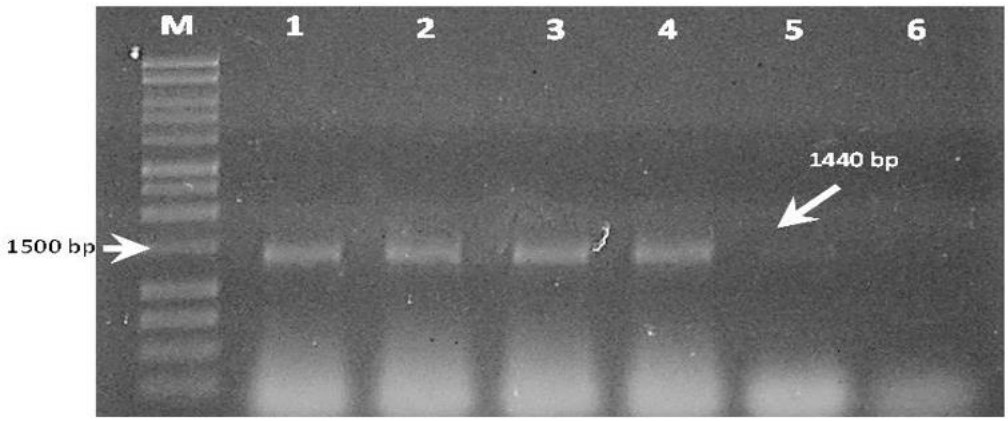

Fig. 4. Agarose gel electrophoresis showing the nested -PCR amplification of 1440 bp of DNA amplicons from infected lily plant using the F0/R1 primer pair amplifying the 16S rRNA gene. M= 1kb DNA ladder, 1-5 infected samples, and 6 = healthy lily plant.

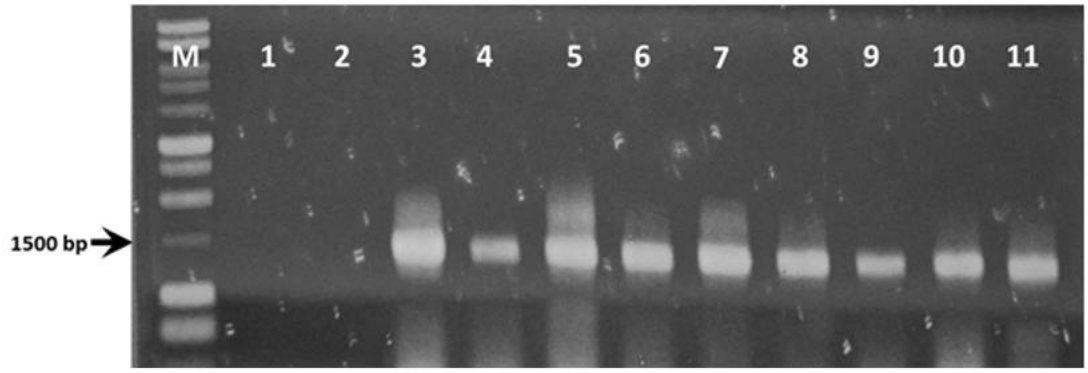

Fig. 5. Agarose gel electrophoresis showing the amplification of 1500 bp of DNA amplicons of the (16S rRNA-spracer region) operon with the specific P1/AYint primers for detection of members of the Aster yellows group. M= 1kb DNA marker, 1= -ve control; no DNA templet, 2 = a healthy lily plant. Samples 3-11 = infected lily plants.

Fig. 6. Recovered lily plants from phytoplasma-induced symptoms after treatment with tetracycline hydrochloride antibiotic.

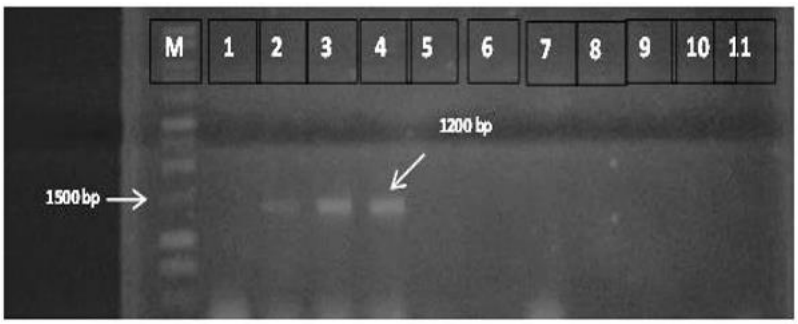

Fig. 7. Agarose gel electrophoresis showing the amplification of 1200 bp of DNA amplicons of the 16S rRNA gene with the universal F2n/R2 primers. M= 1kb DNA marker, 1= healthy control; 2 -11, tested lily plant for the presence of phytoplasma after antibiotic treatment.

DISCUSSION

The developed symptoms on lily plants in this study are typical for phytoplasma symptoms described upon infection of several crops with AY phytoplasma (Lee *et al.*, 2003. The Leaf epinasty, malformation, leaf senescence, stunting, and tissue decline symptoms are due to hormonal imbalances in biosynthesis, conjugation, and transport and signaling in the plant (Hogenhout *et al.,* 2008; Ding *et al.* 2013; Sandalio *et al.,* 2016; Youssef *et al.,* 2018). The purple top symptoms, or leaf discoloration symptoms, observed in infected lily plants in this study, are characteristic of phytoplasma infection and directly related to anthocyanin accumulation as reported previously by Himeno *et al.* (2014) who found that mutant plants with anthocyanin deficiency did not form purple top symptoms upon phytoplasma infection.

Molecular diagnosis of the AY phytoplasma in lily, in this study, was based on using the universal primers P1/P7 and F2n/R2 which were used by several investigators for the detection of phytoplasmas in host plants as well as in their insect vectors (Lee *et al.;* 1991, 1992; Smart *et al.,* 1996). The primer pair F0/R1 tested positive for the preferential amplification of the AY phytoplasmas (Davis and Lee, 1993). Both F2n/R2 and F0/R1 primer pairs were designed from the 16S rRNA sequence of the Michigan Aster yellows phytoplasma (MIAY) (Lee *et al.,* 1993; Davis and Lee, 1993). The specific P1/Ayint primer pair was designed to amplify part of the highly variable separating region (SR) of the rRNA operon and the 16S RNA gene and was used for the specific detection of AY phytoplasmas (Smart *et al.*, 1996; Youssef *et al.,* 2018). Therefore, the DNA amplicons resulting from using these universal, and specific primer pairs in this work confirm the presence of phytoplasma from the AY group in the infected lily plants.

In the present study, spraying infected lily plants with *Ca*. P. astris with tetracycline hydrochloride at 250 ppm) was successful in eliminating the phytoplasma from 70 % of the tested plants. In different applications of antimicrobials to control phytoplasma, Tanno et al. (2018) used an in vitro plant-phytoplasma co-culture system to examine the effectiveness of over 40 antimicrobias, added to tissue culture media, against cuttings of Garland chrysanthemum plants (*Glebionis coronaria*) infected with the wild-type line of *Ca.* P. asteris onion yellows strain (OY-W). They found that both tetracycline hydrochloride (20 ppm) and rifampicin (100 ppm) were the only antimicrobials successful in eliminating phytoplasmas completely from treated plants. Although those authors attained better control of phytoplasma, with lesser antimicrobial concentration than ours, it is not worth mentioning that the application of antibiotics through spraying is inexpensive, swift, and spares all the efforts and costs paid for building a sophisticated system of tissue culture, per se, and very convincing

for developing countries.

Conclusion:

Because lily plants are vegetatively propagated, the spread of phytoplasma of lily over neighboring areas may represent a threat to other susceptible ornamental and vegetable crops in Egypt. Strict quarantine measures should be followed through testing imported ornaments from abroad.

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