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Occurrence and incidence of phytoplasmas of the 16SrII-D subgroup on solanaceous and cucurbit crops in Egypt

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Abstract Symptoms reminiscent of phytoplasma infection were observed in four provinces (governorates) of Egypt in fields of eggplants, tomato plants and squash. Diseased plants exhibited stunting, leaf yellows and flower development abnormalities. PCR amplification of 16SrDNA with phytoplasma-specific primer pairs confirmed the phytoplasma presence. Sequencing and phylogenetic analysis indicated that all phytoplasmas had the same partial 16SrDNA sequence, assigning them to the 16SrII-D phytoplasma subgroup. Disease incidence was about 1% among the 20 squash fields surveyed and equally varied from 4% to 15% in the 20 eggplant fields and in the 40 tomato fields inspected. The widespread distribution of this phytoplasma in annual solanaceous and cucurbit crops suggests a wider plant host range including wild plants that could act as reservoir and insist on the need for a insect vector survey. A finer genetic differentiation of Egyptian 16SrII-D phytoplasma strains from different geographical origins and different host plants should help to better trace such epidemics.

Keywords Tomato · Cucurbit · Eggplant · Phloem-restricted bacteria

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Introduction

The eggplant (*Solanum melongena*), tomato plant (*Lycopersicon esculentum*) and squash (*Cucurbita pepo*) are considered important vegetable crops in Egypt. The cultivated area is estimated about 50,000, 250,000 and 40,000 hectares which produces 250,000, 400,000 and 175,000 tons, respectively in 2009 according to the FAO (2011). These crops are reported to be infected by many viruses, bacteria and fungal diseases in Egypt, but not yet by phytoplasmas. All over the world, phytoplasmas cause a multitude of diseases in several hundred crop species including fruits, vegetables, cereals and trees (McCoy et al. 1989; Lee et al. 2000). The most common symptoms due to phytoplasma infection are yellows, little leaf, stunted growth, witches'-broom, rosetting compact growth of stem, virescence (greening of petals), phyllody (development of small leaves replacing petals or sepals), big bud, sterility of flowers

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(McCoy et al. 1989; Lee et al. 2000). A number of insect species of the order Hemiptera have been demonstrated to be vectors of phytoplasmas (Weintraub and Beanland 2006). Phytoplasmas can also be transmitted through grafting and vegetative propagation through cutting, storage tubers, rhizomes or bulbs (Lee and Davis 1992). An approach to phytoplasma detection and classification has targetted the highly conserved genes coding for the 16S rRNA, and the spacer region between 16S and 23S rRNA (Deng and Hiruki 1991; Lee et al. 1993; Schneider et al. 1995a; Gundersen and Lee 1996). Up to now, numerous hundreds of phytoplasma strains have been classified into 28 groups on the basis of diverse 16S rRNA gene restriction fragment length polymorphism (RFLP) pattern types resolved on wet or in silico (Wei et al. 2007; Lee et al. 2007). Members of the16SrII-D group of the phytoplasma taxonomy formerly known under the now invalidated species name 'Candidatus Phytoplasma australasiae' (White et al. 1998; Firrao et al. 2004) are associated with diseases in economically important crops. These diseases include Papaya yellow crinkle (PpYC), Papaya mosaic (PpM) and Tomato big bud (TBB) in Australia and Pale Purple Coneflower Witches'-Broom (White et al. 1997; Schneider et al. 1999; Pearce et al. 2011). Up to now, only phytoplasmas of the group 16SrI has previously be reported to cause periwinkle virescence in Egypt (strain EPV for Egyptian periwinkle virescence) (Omar et al. 2008). In the neighbouring country of Sudan, faba bean phyllody was a devastating disease during the nineteen-eightees and was later classified as member of the 16SrDNA subgroup II (Cousin et al. 1989; Saeed et al. 1993; Saeed et al. 1994; Schneider et al. 1995b). Elsewhere, Brinjal little leaf phytoplasma disease (BLL-Bd) was reported from Bangladesh, India, Brazil and mainly associated with 16SrII phytoplasma groups strains (Mitra 1988; Boiteux et al. 1994; Schneider et al. 1995b; Siddique et al. 2001). In Egypt, phytoplasma associated with malformation of mango fruits were observed by electron microscopy but were not characterized (El-Banna and El-Deeb, 2007). The objective of this study is to investigate the incidence and etiology of tomato, eggplant and cucurbit disorders recently observed in Egypt which are reminiscent of phytoplasma infection, and also to characterize possibly associated phytoplasmas.

Materials and methods

Survey of phytoplasma

In four governorates of Egypt (Banisweef and Elminia, central Egypt left Nile river bank; Elsharkia and Ismailia, north-eastern Egypt Nile delta), eighty fields of tomato plants, eggplants and squash were randomly selected and inspected from June to October. In Banisweef (Beni Suel) and Elminia (Mynia) governorates, the survey was conducted in tomato fields. In Elsharkia (Sharkia) and Ismailia governorates surveys were carried out in tomato, eggplant and squash fields. Ten fields of tomato plants were selected in each four governorates and 10 fields of eggplants and squash were selected in Ismailia and Elsharkia (Table 1). Incidence of symptom expression (% of plants with phytoplasma symptoms) was estimated for each field by visual inspection of 1,000 plants following a W pattern (crossing the rows) as a sampling procedure. Symptomatic plant samples of eggplant, tomato and squash were collected stored in plastic bags and transferred to the department of plant pathology, Faculty of Agriculture, Kafrelsheikh University. Samples were kept at 4°C until DNA extraction.

DNA extraction

Plant DNA was extracted from 0.5 g of the mid-vein of 140 symptomatic plants (80 tomato, 40 eggplant and 20 squash) and 3 symptomless plants of each 3 species which were all first crushed in liquid nitrogen with a mortar and pestle. DNAs were extracted using DNeasy® Plant Mini Kit (Qiagen, Valencia, CA, USA) according to manufacturer's instructions and the CTAB (cethyl-trimethyl-ammonium bromide) extraction protocol (Maixner et al. 1995).

PCR amplification of 16S rDNA gene

The primer pairs P1 and P7 were used to prime the amplification of a 1.8 kb product of 16S ribosomal RNA (rRNA) gene, the spacer region between the 16S and 23S rRNA gene, and the beginning of the 23S rRNA gene regions of the phytoplasma genome (Deng and Hiruki 1991; Schneider et al. 1995a). After the initial denaturation step at 94°C for 3 min, PCR

Table 1 Incidence of Egyptian phytoplasmas on tomato, eggplant and squash samples collected from different regions from open field during 2010

Governorate	Crops	Visual inspection Symptomatic/total[a]	% diseased plants (min/max)	PCR Positive/ tested plants
Elsharkia	Tomato	860/10000	8.60 (5.6/9.5)	20/20
	Eggplant	1442/10000	14.42 (10.4/16.6)	20/20
	Squash	115/10000	1.15 (0.7/1.8)	10/10
Ismailia	Tomato	1040/10000	10.40 (7.8/11.3)	20/20
	Eggplant	1540/10000	15.40 (10.2/18.6)	20/20
	Squash	136/10000	1.36 (0.8/2.2)	10/10
Banisweef	Tomato	564/10000	5.64 (2.1/6.2)	20/20
Elminia	Tomato	432/10000	4.32 (3.1/5.3)	20/20

[a]Ten fields were inspected for each crop in each governorate

was performed for 34 cycles which were conducted in an automated thermal cycler (GeneAmp® PCR System 9700), each at 94°C for 1 min, 55°C for 1 min and 72°C for 2 min, followed by a final extension step at 72°C for 7 min. Each PCR mixture (40 µl) contained 25 ng of total nucleic acid plus 20 pmol of each primer, 1.0 unit of Taq DNA polymerase (Qiagen, Hilden, Germany) and a final concentration of 0.2 mm of dNTP, 2.0 mM MgCl2 and 1X PCR buffer. the PCR products obtained in the first amplification were diluted with sterile distilled water (1:20) and 1 µl of each dilution was used as DNA template for amplification by nested PCR using primer pair fU5/rU3 , which was used for amplification of the 881 bp fragment of the phytoplasma16S-rDNA (Seemüller et al. 1994). The PCR products were separated on 1.5% agarose gels with 1-kb plus molecular weight marker (Invitrogen, Carlsbad, California, USA).

Nucleotide sequencing and analysis

Specific fU5-rU3 nested-PCR products of the eighteen Egyptian isolates amplified from ten infected tomato plants, three eggplants and five squash plants belonging to different regions were directly sequenced. The nucleotide sequences were determined in both directions and performed by Beckman Coulter Genomics, Takeley, UK, on ABI PRISM capillary sequencing instruments. Raw sequence chromatograms were assembled and edited using GAP4 (Bonfield et al. 1995) to correct ambiguous bases or remove low quality stretches from the termini of the sequences. Homologies to known sequences were detected using the BLASTN algorithms (Altschul et al. 1997) against the non-redundant GenBank database at http://www.ncbi.nlm.nih.gov/blast. Multiple alignments were performed using ClustalW (Thompson et al. 1994) and the phylogenetic analyses were conducted with MEGA4 using the neighbour joining method (Tamura et al. 2007).

The partial fU5-rU3 16Sr-DNA gene sequence of Egyptian phytoplasma isolates have been deposited in the EMBL/GenBank/DDBJ databases under accession numbers listed on Fig. 2. The 16S-rDNA gene sequences of different groups of phytoplasmas used for comparisons were retrieved from GenBank (www.ncbi.nlm.nih.gov) are shown in Fig. 2.

Results and discussion

Symptomatology of the diseases

Naturally infected eggplant plants showed severe symptoms such as malformed leaves, virescence (excessive greening of floral tissues) and phyllody (leaf-like structure). The diseased plants did not produce any fruits and appear higher than the non-infected plants (Fig. 1a & b). Tomato plants in general showed stunting, yellowing or purplish leaves, intense proliferation of laterals buds, formation of hypertrophic calixes, virescence, inhibition of anther and ovary formation and fusion of petals leading to big bud abnormalities. Plant apices generally lacked leaves and the youngest leaves were very small, thick and distorted (Fig. 1d & e). Conversely, the diseased squash plants symptoms only corresponded to stunting and virescence (Fig. 1g). Obvious examples of

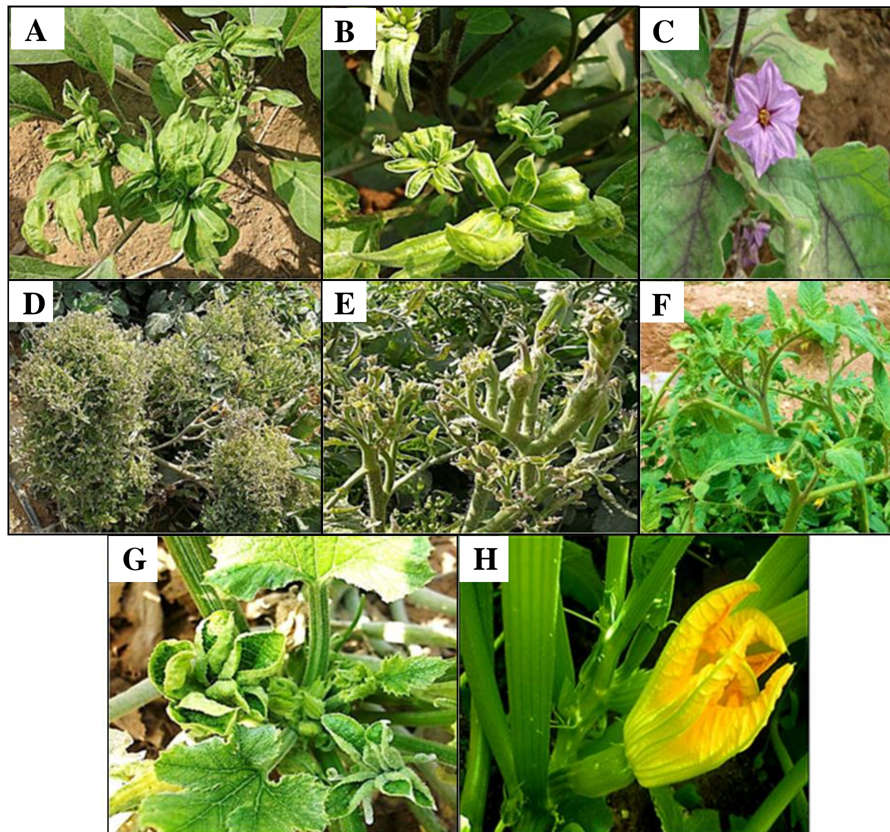

Fig. 1 Pictures of natural symptoms on eggplant; (a): showing phyllody, (b): displaying virescence. (c): healthy eggplant. Natural symptoms on tomato; (d): stunting and proliferations and small purplish leaves (e): hypertrophic calyxes (big bud). (f): healthy tomato plant. Natural symptoms on squash; (g): stunting and virescence. (h): healthy squash flowers

flower deformities are the development of green flowers and the loss of usual flower pigment (virescence), the development of floral organs into leafy structures (phyllody), which were associated to plants infected by stolbur-disease, and in a number of cases, malformations of the stamens and carpels leading to plant sterility (Cousin and Abadie 1982). Similarly, such symptoms have been observed in periwinkle (Catharanthus roseus) or tomato plants infected by the isolate Pyrénées orientales (PO) of stolbur phytoplasma (16SrXII-A subgroup) and aster yellow phytoplasma (16SrI group) (Jarausch et al. 2001; Pracros et al. 2006; Omar et al. 2008). In 2010, these symptoms, prevailing in a large scale on eggplant, tomato and squash plants in the different surveyed governorates, were reminiscent of phytoplasma infection. So, an extensive survey was undertaken to determine the incidence of these disease in Egypt and isolate and investigate a possible association with phytoplasmas infection.

Incidence of the diseases

Disease was about 1% among the 20 squash fields, for instance 1.15% in Elsharkia and 1.36% in Ismailia. In tomato fields, symptoms incidence ranged from 4.32% in Elminia to 10.4% in Ismailia. Eggplant fields were more heavily affected with disease incidence reaching about 15% in both Elsharkia and Ismailia.

DNA amplification

Target DNA of the approximate expected size of 1.8 kbp was amplified using primer pair P1/P7 specific of phytoplasma 16S rRNA from all symptomatic plants of eggplant, tomato plants and squashes as a the first PCR (data not shown). The same primers failed to produce any PCR products when the DNA from healthy plants was used as control. Nested PCR assays with primer pair fU5/rU3 were performed from total DNAs of ten tomato plants (Elminia-1, Elminia-2, Banisweef-1, Banisweef-2,

Banisweef-3, Elsharkia-1, Elsharkia-6, Elsharkia-7, Ismailia-5 and Ismailia-6), three eggplants (Elsharkia-5, Ismailia-3 and Ismailia-4) and five squashes (Elsharkia-2, Elsharkia-3, Elsharkia-4, Ismailia-1 and Ismailia-2). Again, PCR products of the expected size (about 881 bp) were amplified from all symptomatic plants of eggplant, tomato plants and squashes as a the first PCR. The same primers failed to produce any PCR products when the DNA from healthy plants was used as control.

More precisely, surveys performed visually from June and October 2010 to detect diseases associated to phytoplasmas within tomato, eggplant and squash plants showing symptoms of phytoplasma, were confirmed by testing of 80 tomato, 40 eggplant and 20 squash symptomatic plants from the surveyed regions by 16SrDNA PCR and nested PCR. All symptomatic plants were positive (Table 1). This confirmed that in Elsharkia and Ismailia governorates, the incidence of phytoplasmas was 14.42% and 15.40% in eggplant, 8.60% and 10.40% in tomato and 1.15% and 1.36% in squash infected fields, respectively (Table 1). Although, the tomato, eggplant and squash fields were very close to each others in Elsharkia and Ismailia governorates, the incidence of phytoplasma varied from crop to crop with a lower incidence on Cucurbitacae than on Solanaceae. That it was higher in eggplant fields than tomato and squash fields may be due to a higher level of transmission by insect vectors. This certainly resulted from an enhanced insect vector attraction or reproduction on eggplant or a different infectious ability of insect(s) vector(s). The incidence was 5.64% and 4.32% in tomato fields in Banisweef and Elminia governorates, respectively (Table 1). It was lower than the incidence of phytoplasma on tomato and eggplant crops in Elsharkia and Ismailia governorates which were 8.6% and 10.4% respectively (Table 1). The existence of possible wild plant reservoirs, possibly unevenly distributed or controlled, could also explain such difference in geographical disease incidence. Also climate differences between the North of Egypt (Elsharkia and Ismailia) and central Egypt (Elminia and Baniwwef) may influence such variations in disease incidence.

Sequences and phylogenetic analysis

Eighteen 16SrDNA fU5/rU3 nested-PCR products were sequenced and compared between each other and with other phytoplasma 16S-rDNA of groups and subgroups reported in Genbank. The Egyptian phytoplasma isolates in tomato were given the name tomato phyllody phytoplasma (TPP), in eggplant, eggplant phyllody phytoplasma (EPP) and in squash, squash virescence phytoplasma (SVP). The results revealed that all Egyptian phytoplasma isolates were 100% identical, as well as with Papaya yellow crinkle phytoplasma formerly described as 'Ca. P. australasiae' (White et al. 1997; White et al. 1998), which belongs to the 16SrII-D subgroup. All of them contained an additional tru9I restriction site at position 604 of the fU5rU3 sequence by comparison to members of the subgroup II-B and 16SrII-C and lack an TaqI restriction site present at position 431 of the fU5rU3 sequence of members of the subgroup 16SrII-A. These two differences reported to be characteristics of the subgroup 16SrII-C (Khan et al. 2002) are located on fU5rU3 sequence which can therefore be used for the assignment to the subgroup 16SrII-D. All the sequences from Egyptian samples shared 99.9% identity with peanut witches'-broom phytoplasma which corresponds to 16SrII-A subgroup. Lower identities of 98.8% and 97.8% were with 'Ca. P. aurantifolia' of the 16SrII-B subgroup (Accession no. U15442) and 'Ca. P. brasiliense' of the 16SrXV-A subgroup (Accession no. AF147708) respectively. This was awaited as 16SrII and 16SrXV groups represent phylogenetically related phylla (Montano et al. 2001). The lowest identity (85.7%) was with 'Ca. P. caricae' of the 16S rDNA-XVII-A subgroup (Accession no. AY725234).

The phylogenetic analysis of all Egyptian tomato phytoplasma isolates showed they clustered together with Papaya yellow crinkle phytoplasma of 16SrII-D subgroup (White et al. 1998) and Peanut witches'-broom phytoplasma of 16SrII-A subgroup (Gundersen et al. 1994) as well as with the Eggplant phyllody phytoplasma Nubaria isolate (Accession no. FN257482), which was previously classified as 16SrII-D subgroup (Omar, unpublished) (Fig. 2). The phylogenetic analysis also confirmed that the Egyptian tomato plant, eggplant and squash phytoplasmas constituted a monophyletic cluster of African-Australasian origin supported by a bootstrap value of 100. The phytoplasma ‘Candidatus species’ most closely related to Egyptian phytoplasma isolates were 'Ca. P. aurantifolia' and 'Ca. P. brasiliense' (Fig. 2) (Zreik et al. 1995; Montano et al. 2001). As

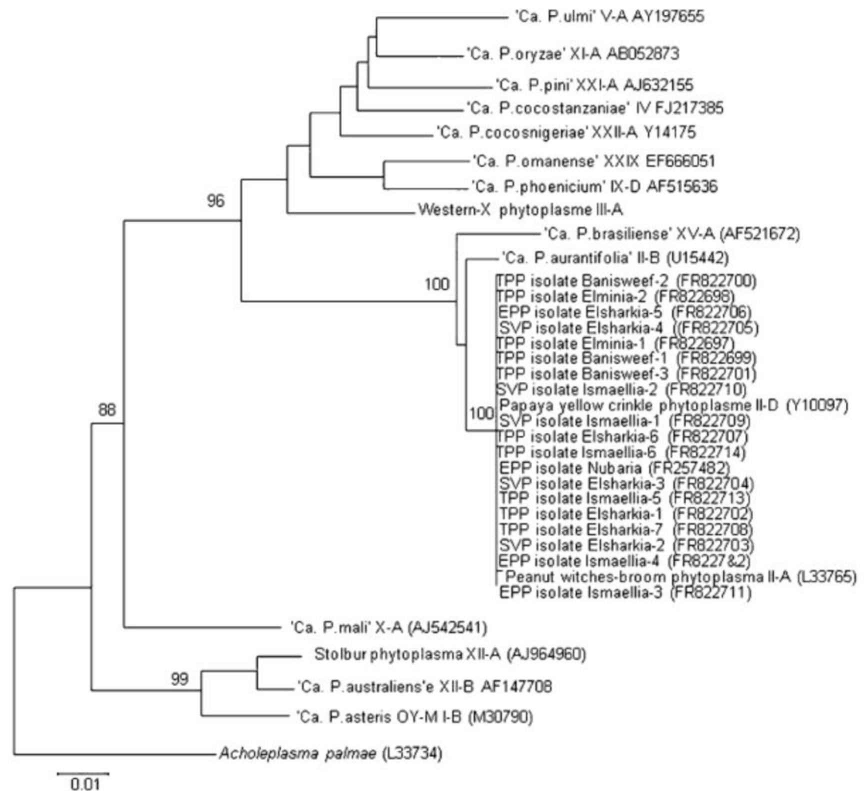

Fig. 2 Phylogenetic tree constructed by the neighbour-joining method, showing the phylogenetic relationships for the partial 16S rRNA nucleotide sequences for all Egyptian phytoplasma samples surveyed, compared with representatives from other 16S rRNA groups. GenBank accession numbers for published sequences are shown in parenthesis alongside the names of the isolates. Bootstrap values greater than 50% (expressed as percentages of 1000 replicates) are shown next to the branches. Phylogenetic analyses were conducted in MEGA4

a consequence of their total 16S identity, the phylogenetic position of Egyptian phytoplasma isolates could not be correlated to their geographical origin. More variable genetic markers, such as non ribosomal genes are needed in order to allow differentiation of 16SrII-D Egyptian phytoplasmas at the strain level. Studies on specificity to plants and geographical distribution would also benefit from such markers.

In the present study 16SrII-D phytoplasmas are proved to infect tomato, eggplant and squash plants. Recently in Australia, they were reported to infect papaya, tomato (White et al. 1998) and Pale Purple Coneflower (Pearce et al. 2011) but not eggplant and squash, and the strains have not yet been genetically differentiated.

Phytoplasmas belonging to different groups have been reported to be associated to disease in cucurbits (Streten et al. 2005; Montano et al. 2000; Montano et al. 2006; Montano et al. 2007). Six different taxonomic phytoplasma groups infect tomato plants wordwide (Blancard 2009; Davis et al. 1997; Shaw et al. 1993; Hiruki and Wang 2004; Anfoka et al. 2003; Choueiri et al. 2007; Garnier 2000; Lee et al. 1993; Arocha et al. 2007; . Eggplant diseases have also been reported to be associated with phytoplasmas of different groups ; Schneider et al. 1995b; Sertkaya et al. 2007; Kelly et al. 2009; Al-Subhi et al. 2011; Amaral-Mello et al. 2010).

In conclusion, our analyses demonstrated that the Egyptian phytoplasma isolates causing diseases of tomato plant, eggplant and squash are members of the 16SrII-D clade of the phytoplasma 16Sr-DNA RFLP classification. They have identical partial 16SrDNA sequences. These isolates are widespread in the most three important vegetable crops in Egypt. This study also suggests that these Egyptian isolates are widespread in Egypt and certainly propagate from crop to crop or from wild reservoir to crop by insect vectors. Further entomological and botanical investigations are required to identify the insect vectors responsible for the phytoplasma transmission in the country and to determine its plant and insect host range. In addition, finer genetic differentiation of isolates will be needed

to trace the geographical and dynamics of its epidemics.

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