

Detection of Phytoplasmas Associated with Tomato Witches' Broom and Big Bud Disease in Egypt

Behiry, S. I.

Agricultural Botany Department, Faculty of Agriculture Saba Basha, Alexandria University, Egypt.



ABSTRACT

Symptoms of witches' broom were observed on tomato plants in some fields in Aswan governorate, Egypt. Molecular investigation was done with phytoplasma-detection primer P1/P7 to amplify a fragment of the 16S rRNA gene plus spacer region and beginning of 23Sr gene. RFLP analyses and sequencing of DNA fragments amplified by R16F2n/R2 primers indicated that these phytoplasmas are closely related and could be classified in the 16SrII group. Phylogenetic analyses of 41 accessions of 16S ribosomal RNA gene sequences of 'Candidatus phytoplasmas' comprising the strain from Egypt and representative strains from GenBank confirmed that the phytoplasma from tomato cluster with other strains all classified in subgroup 16SrII-D. Therefore, it could be useful to use the RFLP methodology in rapid and specific screenings of this phytoplasma presence in Egyptian tomato plants.

Keywords: tomato, phytoplasmas, 16SrII, RFLP

*Corresponding author: said.behiry@alexu.edu.eg

INTRODUCTION

Tomato (*Solanum lycopersicum* L.) is an important crop in all Egypt Governorates, tomato is a particularly important crop for small farmers, in Egypt which has a total annual production of 7943285 tons, in 2016 according to the FAO (2016). Tomato phytoplasma diseases are associated with a wide variety of symptoms, including yellowing (Tapia-Tussell *et al.* 2012), witches' broom, virescence, flower malformations including big bud and have been distinct in a lot of ribosomal subgroups worldwide (Shaw *et al.* 1993; Marcone *et al.* 1997; Lee *et al.* 1998; Anfoka *et al.* 2003; Arocha *et al.* 2007; Vellios and Lioliopoulou 2007; El-Banna *et al.* 2007; Singh *et al.* 2012; Du *et al.* 2013; Xu *et al.* 2013; Salehi *et al.* 2014). The 16SrII group of phytoplasmas in Egypt were noticed previously associated with tomato big bud (El-Banna *et al.* 2007; Omar and Foissac 2012).

Molecular techniques such as polymerase chain reaction (PCR), restriction fragment length polymorphism (RFLP) and sequence analysis have been used as tools for phytoplasma differentiation and strain characterization. (Lee *et al.* 1998) In this study we work on the group affiliation and characterization of tomato phytoplasma strains to develop a rapid field screening to tomato phytoplasmas disease in Egypt.

MATERIALS AND METHODS

Samples collection

Symptoms of witches' broom and abnormal united calyx fragments noticed on tomato plants (big bud) (Fig. 1) were collected from fields in Aswan Governorate, Egypt.

Molecular identification and characterization.

DNA was extracted from 1 g of the mid-drip of 11 tomato symptomatic plants using a CTAB (cetyl-trimethylammonium bromide) extraction protocol (Angelini *et al.* 2001). The PCR primer pair P1/P7 was used to prime a 1800 bp fragment consisting of the 16S rRNA gene, the 16S-23S spacer region and partial region of 23S rRNA gene (Schneider *et al.* 1995). A dilution from P1/P7 PCR amplification was used for nested PCR with primer pair R16F2n/R2 to magnify a fragment of 1.25 kb in the 16S rRNA gene (Gundersen and Lee 1996). After the initial denaturation step at 95°C for 3 min, 35 cycles were

conducted in thermal cycler (Techne, England) each at 94 °C for 45 sec., 55 °C for 30 sec., extension at 72 °C for 1 min, and final elongation at 72 °C for 7 min. PCR reaction (25 µl) contained 1 µl of DNA preparation plus 0.5 µl of each primer, 1 µl of stained *Taq* DNA polymerase (Bioline, Korea), 2 µl of dNTPs, 2 µl of 10X PCR buffer and the final volume obtained by adding molecular grade water. Phytoplasma positive control, sweet potato little leaf (SPLL, 16SrII-A) engaged in this study from phytoplasma reference strains propagated in periwinkle (Bertaccini, 2015). Negative controls; were added as samples free from DNA in all PCR amplifications.

RFLP analyses of nested R16F2n/R2 primed bands were conducted using about 200 ng template DNA for each sample that was restriction enzymes digested separately with the *Mse*I, *Rsa*I and *Taq*I (Biolabs, New England) instructed by the manufacturer. The RFLP digested products were then electrophoresed through a 6.7 % acrylamide gel stained with ethidium bromide. Fragments of DNA were then visualized with transilluminator.

Positive R16F2n/R2 amplified bands were subjected to sequence after purification at Macrogen, Inc., Korea. The obtained sequences were analysed by MEGA 6.06 software (Tamura *et al.* 2013) and the consensus sequence was then employed for phylogenetic analyses using sequences retrieved in the GenBank database from taxonomically representative 'Candidatus phytoplasma' strains plus *Acholeplasma laidlawii* as outgroup. the Neighbor-Joining method was used to deduce the genetic evolutionary history (Saitou and Nei 1987).

RESULTS AND DISCUSSION

Typical symptoms of tomato were previously noticed to happen in neighboring countries; Jordan, Italy, Greece (Alivizatos 1989; Shaw *et al.* 1993; Del Serrone *et al.* 2001). Out of the 11 symptomatic tomato samples resulted three positive in direct amplification with P1/P7 primers and also in nested PCR with R16F2n/R2 producing the expected bands of about 1,800 bp and 1,245 bp respectively (Fig. 2). The RFLP analyses of those amplicons and of amplicon obtained from reference strain SPLL belonging to different phytoplasma subgroup-A,

indicated that the phytoplasma infecting tomato belong to 16SrII subgroup –D (Fig. 3). One of the strains designed Tomato Egypt (To-1), was sequenced and the 1,104 bp sequence obtained after alignment was submitted to Genbank under the accession number (MH981943). Phylogenetic analyses performed on 16S ribosomal gene comparing the 1,104 bp of the 16S rRNA gene of To-1 strain to 41 phytoplasma strains enclosing major

‘*Candidatus* Phytoplasma’ species available and representatives available 16SrII subgroups allow to confirm that To-1 strain clusters with the other phytoplasmas classified in 16SrII-D subgroup, that are clustering together and are phylogenetically differentiable from the other ribosomal subgroups in group 16SrII with a good branch support (Fig. 4).



Fig. 1. Symptoms on a tomato shoot infected by 16SrII phytoplasmas.

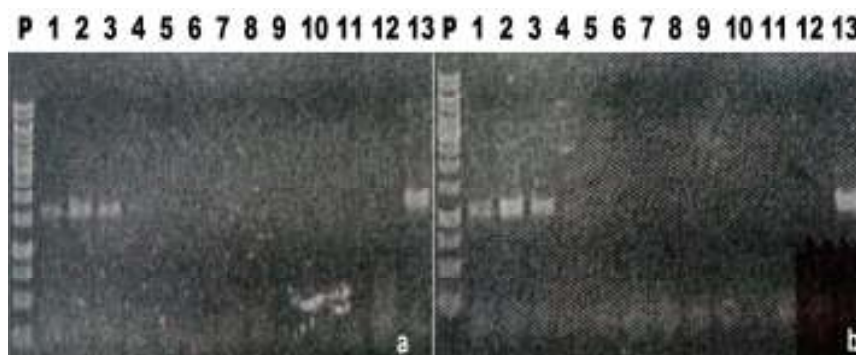


Fig.2. Agarose 1% gel with results of direct PCR amplification using in a) the primers P1/P7 with tomato samples 1, 2,3,4,5,6,7,8,9,10,11 ;12, SDW; 13, sweet potato little leaf (SPLL, 16SrII-A); in b) the primers R16F2n/R2 with tomato samples 1, 2,3,4,5,6,7,8,9,10,11 ;12, SDW; 13, sweet potato little leaf (SPLL, 16SrII-A).P, 1 kb DNA ladder with different sizes in base pairs from top to bottom of 10,000; 8,000; 6,000; 5,000; 4,000; 3,000; 2,500; 2,000; 1,500; 1,000; 750; 500 and 250.

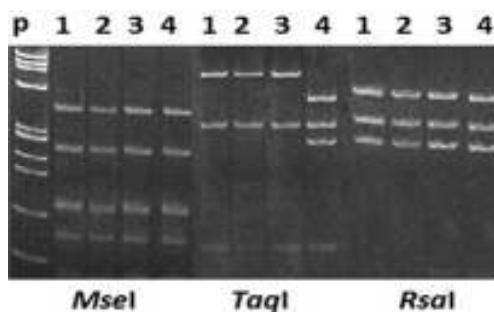


Fig. 3. The digestion restriction fragment length polymorphism patterns of R16F2n/R2 primer pair products shown on acrylamide gel with three restriction enzymes listed at the bottom of the figure from Egyptian tomatoes (1, 2 and 3) and 4, sweet potato little leaf (SPLL, 16SrII-A). P, marker phiX174 *Hae*III with different sizes from up to down of 1353, 1078, 872, 603, 310, 281, 271, 234, 194, 118 and 72 bp

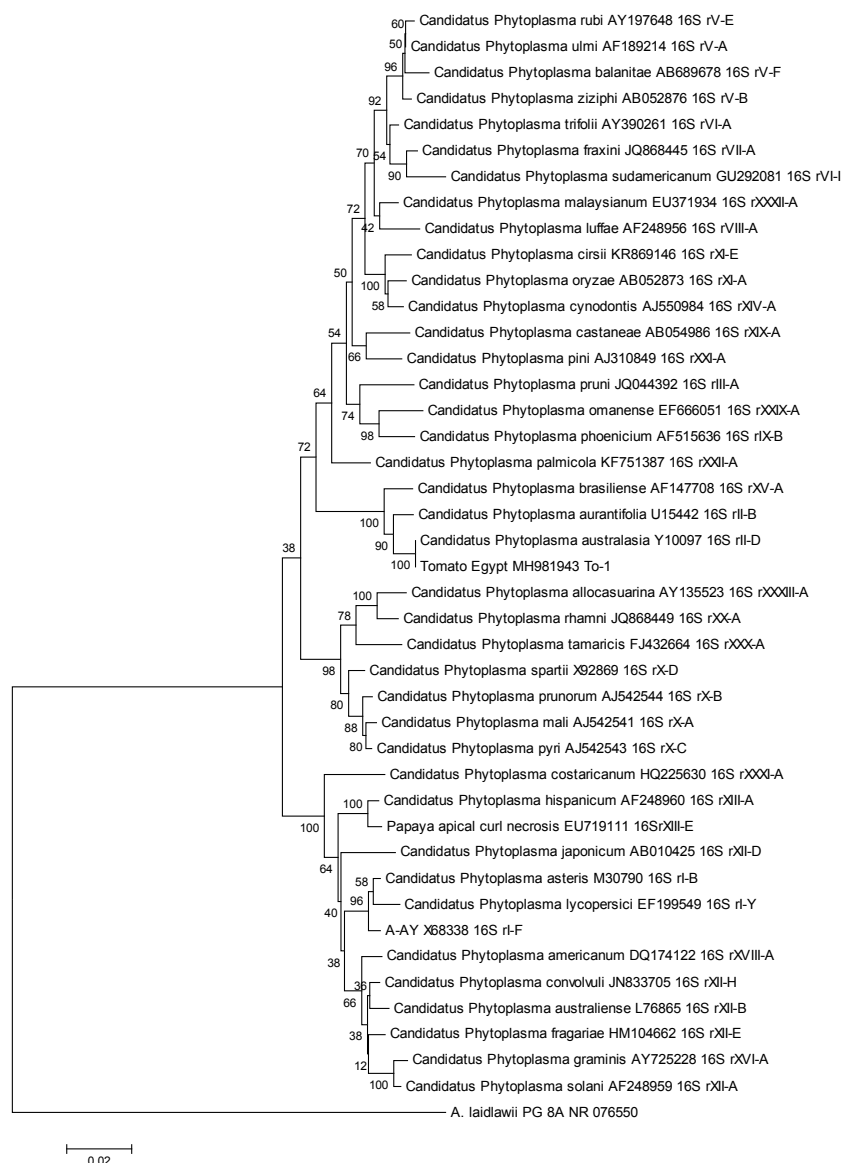


Fig. 4. Evolutionary relationships on 16S rRNA gene sequences (1,104 bp) of phytoplasmas closely related to ‘*Ca. P. australasia*’ strain tomato Egypt. Genbank numbers of ribosomal sequences used are written after the strain acronyms. *A. laidlawii* was used as out group to root the tree.

The results of this survey revealed that the 16S ribosomal sequences of Egyptian phytoplasma strain examined in this survey were 100% identical to each other's and to those of '*Ca. P. australasia*' (White *et al.* 1998), affiliated to the 16SrII-D subgroup. Phytoplasmas identified as belonging to 16S rII-D subgroup have been reported in central left Nile river bank and north-eastern Nile delta governorates in Egypt associated with diseases in vegetable crops (Omar and Foissac 2012) indicating that this phytoplasma is very likely quite spread in Egyptian cultivation also other than tomato.

Further data required to determine which other hosts associated with the same phytoplasma detected in tomatoes to avoid spreading of the disease to other crops or areas.

REFERENCES

- Alivizatos, A.S. (1989). Occurrence and distribution of tomato stolbur in Greece. Proc. Conf. Plant Pathogenic Bacteria, 7th. Budapest, Hungary
- Anfoka, G.H.A.; A.B. Khalil and I. Fattash (2003). Detection and molecular characterization of phytoplasma associated with big bud disease of tomatoes in Jordan. J. Phytopath. 151:223-227.
- Angelini, E.; D. Clair; M. Borgo; A. Bertaccini and E. Boudon-Padieu (2001). Flavescence dorée in France and Italy - Occurrence of closely related phytoplasma isolates and their near relationship to Palatinate grapevine yellows and an alder yellows phytoplasma. Vitis 40, 79-86.

- Arocha, Y.; O. Antesana; E. Montellano; P. Franco; G. Plata and P. Jones (2007). 'Candidatus Phytoplasma lycopersici', a phytoplasma associated with 'hoja de perejil' disease in Bolivia. *Int. J. Syst. Evol. Microbiol.* 57(8):1704-1710.
- Bertaccini, A. (2015). <http://www.ipwgnnet.org/collection> [last accessed 30 June 2015]
- Del Serrone, P.; C. Marzachi; M. Bragaloni and P. Galeffi (2001). Phytoplasma infection of tomato in central Italy. *Phytopath. Medit.* 40:137-142.
- Du, Y.; H. Mou; B. Shi; X. Xu and B. Xiang (2013). Molecular detection and identification of a 16SrVI group phytoplasma associated with big bud disease in Xinjiang, China. *J. Phytopath.* 161:870-873.
- El-Banna, M.; M. Om-Hashem; M.S. Mikhail; G.A. Farag and A.M.S. Mohammed (2007). Detection of phytoplasma in tomato and pepper plants by electron microscopy and molecular biology based methods. *Egypt. J. Virol.* 4:93-111.
- FAO (2016) FAOStat. Egypt: Food and Agriculture Organisation of the United Nations.
- Gundersen, D.E. and I-M. Lee (1996). Ultrasensitive detection of phytoplasmas by nested-PCR assays using two universal primer pairs. *Phytopath. Medit.* 35:144-151.
- Lee, I-M.; D.E. Gundersen-Rindal; R.E. Davis and I. M. Bartosz (1998). Revised classification scheme of phytoplasma based on RFLP analyses of 16S rDNA and ribosomal protein gene sequences. *Int. J. Syst. Bacteriol.* 48: 1153-1169.
- Marcone, C.; A. Ragozzino and E. Seemüller (1997). Detection and identification of phytoplasmas infecting vegetable, ornamental and foliage crops in southern Italy. *J. Pl. Pathol.* 79:211-217.
- Omar, A.F. and X. Foissac (2012). Occurrence and incidence of phytoplasmas of the 16SrII-D subgroup on solanaceous and cucurbit crops in Egypt. *Eur. J. Pl. Pathol.* 133:353-360.
- Saitou, N. and Nei, M. 1987. The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Mol. Biol. Evolut.* 4: 406-425.
- Salehi, E.; M. Salehi; S.M. Taghavi and K. Izadpanah (2014). A 16SrII-D Phytoplasma strain associated with tomato witches' broom in Bushehr province, Iran. *J. Crop Prot.* 3(3):377-388.
- Schneider, B.; E. Seemüller; C.D. Smart and B.C. Kirkpatrick (1995). Phylogenetic classification of plant pathogenic mycoplasma like organisms or phytoplasmas. In S. Razin & J. G. Tully (Eds.), *Molecular and diagnostic procedures in mycoplasmaology* (pp. 369-380). San Diego, CA: Academic.
- Shaw, M.E.; B.C. Kirkpatrick and D. Golino (1993). The beet leafhopper-transmitted virescence agent cause tomato big bud disease in California. *Pl. Dis.* 77:290-295.
- Singh, J.; A. Rani; P. Kumar; V.K. Baranwal; P.L. Saroj and A. Sirohi (2012). First report of a 16SrII-D phytoplasma 'Candidatus Phytoplasma australasia' associated with a tomato disease in India. *New Dis. Reprt.*: 14.
- Tamura, K.; G. Stecher; D. Peterson; A. Filipski and S. Kumar (2013). MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. *Mol. Biol. Evol.* 30: 2725-2729.
- Tapia-Tussell, R.; A. Suaste-Dzul; A. Cortes-Velazquez; C. Torres-Calzada; A. Quijano-Ramayo; R. Martin-Mex; A. Nexticapan-Garcez and D. Perez-Brito (2012). Molecular characterization of Yucatan tomato phytoplasma (Group 16SrIII). *Afr. J. Biotech.* 11:2169-2177.
- Vellios, E. and F. Lioliopoulou (2007). Detection and characterization of phytoplasmas infecting tomato plants in Greece. *Bull. Insectol.* 60:157-158.
- White, D.T.; L.L. Blackall; P.T. Scott and K.B. Walsh (1998). Phylogenetic positions of phytoplasmas associated with dieback, yellow crinkle and mosaic diseases of papaya, and their proposed inclusion in 'Candidatus Phytoplasma australiense' and a new taxon', *Candidatus Phytoplasma australasia*. *Int. J. Syst. Evol. Microbiol.* 48:941-951.
- Xu, X.; H-Q. Mou; S.F. Zhu; X.L. Liao and W.J. Zhao (2013). Detection and characterization of phytoplasma associated with big bud disease of tomato in China. *J. Pl. Pathol.* 161:430-433.

الكشف الدقيق عن الفيتوبلازما المرتبطة بمرض مكنسة الساحرة وتضخم البرعم في الطماطم في مصر

سعيد ابراهيم عبدالله بحيرى

قسم النبات الزراعى - كلية زراعة سايباشا - جامعة الاسكندرية

لوحظت بعض الأعراض المرضية لمكنسة الساحرة وتضخم البرعم المتسبب عن الفيتوبلازما على نباتات الطماطم المنزرعة بالوجه القبلى بمحافظة اسوان بصعيد مصر وتم التحقق وراثيا من المرض باستخدام بعض الكواشف المتخصصة المباشرة والمتشعبة للكشف عن التتابع الجينى لجين 16Sr RNA واثبتت النتائج أن المتسبب الرئيسى عن المرض محل الدراسة هو نوع من الفيتوبلازما يتبع المجموعة الثانية التصنيفية للفيتوبلازما وبمقارنة التتابعات المتحصل عليها فى بنك الجينات ب41 تتابع خاص بعزلات اخرى من الفيتوبلازما تحت مجاميع تصنيفية مختلفة وتسبب امراض مشابهة او مختلفة أكدت النتائج وكذلك تم استخدام تقنية RFLP المستخدمة فى دراسة الاختلافات بين التتابعات الوراثية الشديدة التشابه باستخدام انزيمات القطع المتخصصة والعشوائية تم معرفة نوع الفيتوبلازما المتسبب عن المرض وتصنيفه الفعلى والذى يتبع 16SrII-D