

## Evidence of a Mixed Infection of *Candidatus Phytoplasma Trifolii* and a Begomovirus in Eggplant (*Solanum melongena*)

Jitender Singh<sup>1</sup>, Aastha Singh<sup>1</sup>, Pankaj Kumar<sup>1</sup>,  
Anchal Rani<sup>1</sup>, V.K. Baranwal<sup>2</sup> and Anil Sirohi<sup>1</sup>

<sup>1</sup>College of Biotechnology, Sardar Vallabhbhai Patel University of Agriculture and Technology, Meerut, India - 250110, India.

<sup>2</sup>Plant Virology Unit, Division of Plant Pathology, Indian Agricultural Research Institute, New Delhi -110 012, India.

(Received: 06 September 2014; accepted: 12 November 2014)

Symptoms associated with eggplant little leaf and leaf curl disease of eggplant was observed in the fields of Meerut district of Western Uttar Pradesh, India. Total DNA extracted from symptomatic and asymptomatic plants was subjected to nested-PCR assay using phytoplasma 16S rDNA primers and generic PCR with universal begomovirus coat-protein (CP) gene primers. Amplicons of expected size were obtained from symptomatic plants in both PCR assays. No PCR amplifications were observed for asymptomatic plants. A multiplex nested PCR assay was also developed by optimizing reaction components and reaction cycling parameters for simultaneous detection of Tomato leaf curl virus (ToLCV) and a phytoplasma. The eggplant infecting phytoplasma was identified as a member of group 16SrVI, "*Candidatus Phytoplasma trifolii*", while the begomovirus showed 99% of CP gene identity with other whitefly transmitted begomoviruses including *Solanum nigrum* and Tomato leaf curl virus (ToLCV). Results indicate that both phytoplasma and begomovirus can co-infect the same host plant and the multiplex PCR provides a sensitive, rapid and low cost method for simultaneous detection of little leaf of eggplant and whitefly-transmitted Tomato leaf curl virus.

**Key words:** Eggplant, Brinjal, Phytoplasmas, Begomoviruses, nested-PCR.

Eggplant (*Solanum melongena* L.) is an important vegetable grown throughout the world. India ranks third in the world in production of eggplant or brinjal, where it is cultivated over 680,000 Ha with a total annual yield of 11,896,000 tonnes (FAOSTAT, 2013). A disease of low incidence affecting approximately 1-5% of plants was observed in eggplant fields of Horticulture Research Centre, Sardar Vallabhbhai Patel University of Agriculture and Technology, Meerut during the month of February, 2012. Symptoms

included yellowing of leaf lamina with upward leaf curl as well as distortion, reduction in internodes, new leaves size reduction, stunted growth and dissemination of flower from plant before onset of fruiting.

Phytoplasmas are nonhelical mollicutes that inhabit the phloem and are causative agents of diseases in several hundred plant species characterized by deformed flowers, plant growth aberrations and yellowing. Phytoplasmas are naturally transmitted by leafhoppers and planthoppers and currently cannot be cultured *in vivo*, therefore these are differentiated and classified on the basis of DNA restriction analysis and sequence analysis of 16S rDNA gene and 16S-23S rRNA intergenic spacer region (Seemuller *et al.* 1998). A number of eggplant diseases associated

\* To whom all correspondence should be addressed.  
Tel.: +91-121-2888534; 91-9410455334  
E-mail: jeets\_80@rediffmail.com

with phytoplasma have been reported earlier, these include Brinjal Little Leaf from Bangladesh and India caused by phytoplasma affiliated to group 16SrI and 16SrVI (Kelly *et al.* 2009; Siddique *et al.* 2001; Wei *et al.* 2008 and Kumar *et al.* 2012). In Japan, a 16SrI group phytoplasma was isolated from eggplant with dwarfing symptoms (Okuda *et al.* 1997) and in Brazil, phytoplasma members of subgroups 16SrIII-B, 16SrIII-J and 16SrIII-U were found to be associated with eggplant giant calyx disease (Mello *et al.* 2011). However, similar symptoms are also induced by begomoviruses group of family *Geminiviridae* (Pratap *et al.* 2011) and presence of whitefly population in the eggplant fields also supported the hypothesis of mixed infection. Plant viruses and phytoplasmas are routinely detected by PCR.

On the other hand the Tomato leaf curl virus (ToLCV) causes one of the most devastating viral diseases of tomato (*Solanum lycopersicum*) which has emerged causing damage and encroaching new areas in tropical and subtropical continents every year. Plant-infecting geminiviruses belong to the family *Geminiviridae* in which Begomovirus is one among the genera possessing both mono- and bipartite genomes that infect especially dicotyledonous plant species (Gronenbor, 2007). The disease is marked by symptoms such as yellowing of leaf lamina with upward leaf curl as well as distortion, reduction in internodes, new leaves size reduction, wrinkle facade, stunted growth, and dissemination of flower from plant before onset of fruiting similar to symptoms induced by phytoplasma. Whiteflies harboring virus can nonspecifically infect a wide spectrum of plant crops and weeds including eggplant, potato, tobacco, pepper, and common bean. Infected plants may be asymptomatic but develop symptoms later leading to enormous economic loss (Melzer *et al.* 2010)

In the case of a mixed infection, PCR detection of all the pathogens with respective primers separately requires more time, cost and effort. Multiplex PCR-based methods are more efficient in which more than one pair of primers are used together in one reaction leading to simultaneous amplification of different sizes of DNA fragments (Tao *et al.* 2012). It reduces cost, saves time and is efficient for detecting mixed infection. Thus the present study was undertaken

to determine the aetiology of the yellow type disease of eggplants and a multiplex nested PCR method has been developed for simultaneous detection of “*Candidatus Phytoplasma trifolii*” and Tomato leaf curl virus (ToLCV).

## MATERIALS AND METHODS

### Plant material

A disease of low incidence approximately 1-5% of plants was observed in eggplant (*Solanum melongena*) fields of Horticulture Research Centre of Sardar Vallabhbhai Patel University of Agriculture and Technology, Meerut (29° 5' 8" N, 77° 41' 47" E) during the month of February, 2012. Samples were collected from symptomatic and asymptomatic plants. Symptoms were yellowing of leaf lamina with upward leaf curl as well as distortion, reduction in internodes, new leaves size reduction (little leaf), stunted growth and dissemination of flower.

### Genomic DNA extraction and PCR conditions for detection of whitefly transmitted begomovirus

To confirm the suspicion of mixed infection, total DNA was extracted from 15 samples from midribs as well as leaves of symptomatic and asymptomatic plants using DNeasy Mini Kit, Qiagen, Germany according to the manufacturer's instructions. The genomic DNA isolated by the above-said method was used as template in PCR for amplification of the coat protein gene of ToLCV. For PCR, one primer set, AV1F: 5'CGAACCGACCAGCAGATATCA3' and AV1R: 5'TTTGATGCATGAGTACAGGCCA'3 specific for the DNA-A component (Ghosh *et al.* 2009), was used for amplification. All the amplifications were performed in 50 µl reaction mixture containing 50 ng of template DNA, 3 unit of Taq polymerase (New England BioLabs, Ipswich, MA, USA), 25 mmol<sup>-1</sup> MgCl<sub>2</sub> (1 µl), 10 mmol<sup>-1</sup> dNTPs (0.5 µl), 0.5 µmol<sup>-1</sup> of each of the forward and reverse primers (1 µl) and 10X reaction buffer (3.0 µl). The amplification was carried out using a thermal cycler (Bio-Rad Laboratories, Hercules, CA, USA). PCR cycling profile consisted of initial denaturation at 94°C, 10 min; followed by 35 cycles of denaturation at 94°C for 30 s; annealing at 56°C for 30 s; and extension at 72°C for 1 min, a final extension step at 72°C for 10 min was carried out to ensure the completion of

amplification of all the target templates. Amplified products were visualized by agarose gel electrophoresis in the presence of ethidium bromide.

#### **Nested PCR assay for detection of phytoplasma**

To test the presence of phytoplasma, 80 ng of purified total DNA was used for PCR amplification. The amplification was carried out in a thermal cycler (Bio-Rad T100; Bio-Rad Laboratories Inc) with 50  $\mu$ l reaction mixture consisting of 2.5 unit polymerase (Invitrogen, Life Technologies, Brazil),  $MgCl_2$  (1  $\mu$ l), 10 mmol<sup>-1</sup> dNTPs (1  $\mu$ l), 0.5  $\mu$ mol<sup>-1</sup> of each of the forward and reverse primers (1  $\mu$ l) and PCR buffer (3.0  $\mu$ l).

Phytoplasma-specific 16S rDNA universal primers P1: 5' AAGAGTTTGATCCTGG CTCAGGATT 3' / P7 5' CGTCCTTCATCGGCTCTT 3' (Deng and Hiruki 1991), which span the 16S rDNA, the 16S-23S spacer and the 52' end of 23S gene, were used in first round of PCR. The PCR conditions employed were denaturation at 94°C for 10 min, 94°C for 45 s, 56°C for 45 s, 72°C for 2 min followed by 30 cycles and final extension at 72°C for 10 min. The product was diluted in a ratio of 1:25 and 2  $\mu$ l of it was used in nested PCR as the template with primer pair R16F2n 5'-GAAACGGTTGCTAAGACTGG-3' / R2: 5'-TGACGGGCGGTGTGTACAAACCC-3' (Lee *et al.* 1998), which covers the 16S rDNA region.

#### **Duplex PCR for detecting co-infection of whitefly-transmitted begomovirus and phytoplasma**

A multiplex PCR assay has been developed to allow the simultaneous amplification of DNA fragments using phytoplasma-specific primers viz. P1/P7 (Deng and Hiruki 1991) universal primer pair for 16S rDNA amplification and nested primer pair R16F2n/R2 (Lee *et al.* 1998) and Tomato Leaf curl virus specific (DNA-A region) primer AV1F / AV1R (Ghosh *et al.* 2009). PCR conditions for this nested multiplex have been optimized ensuring the sensitive detection. Two types of template viz. genomic DNA (50 ng) and amplified diluted (1: 25) PCR product of P1/P7 were used to enable simultaneous detection of the virus and the phytoplasma. Concentrations of  $MgCl_2$ , primer pairs, dNTPs and volume of master mix were optimized. Volume of 10x PCR buffer and Taq DNA polymerase (3 U  $\mu$ l<sup>-1</sup>) was also increased. PCR master mix contained 5  $\mu$ l of 10X PCR buffer, 3  $\mu$ l of 25 mmol<sup>-1</sup>  $MgCl_2$ , 1  $\mu$ l of 100  $\mu$ mol<sup>-1</sup> dNTPs

(BioLab), 0.5  $\mu$ l of 100  $\mu$ mol<sup>-1</sup> of each forward and reverse primers, 1  $\mu$ l of Taq DNA polymerase (3 U  $\mu$ l<sup>-1</sup>) and nuclease-free water. Before adding the Taq polymerase, the other reagents were mixed and the mixture was subjected to denaturation at 94°C for 10 min, 94°C for 45 s, 55°C for 45 s, 72°C for 2 min followed by 30 cycles and final extension at 72°C for 10 min. The first and second PCR products were separated in 1% agarose gel by electrophoresis, after staining with ethidium bromide visualized under ultraviolet light.

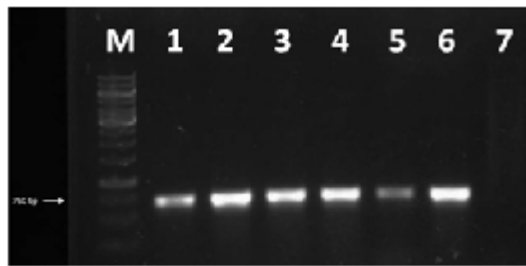
#### **Sequence analysis**

All R16F2n/R16R2 PCR amplified products were purified with PCR Purification Kit (QIAquick Gel Extraction kit, Qiagen, Germany) and directly sequenced at the automated DNA sequencing facility, Department of Biochemistry, Delhi University, South Campus on Applied Biosystems 3730 Genetic Analyzer. Both the 16S rDNA and virus coat-protein sequences were searched for homogenous sequences matching the query sequences in the NCBI nucleotide database with those of the references in GenBank by BLASTn tool (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) (Altschul *et al.* 1990). Sequences obtained were aligned using ClustalW (Thompson *et al.* 1994), and phylogenetic trees were constructed using the Neighbour-joining method with the program MEGA version 5.0 (Tamura *et al.* 2004).

## **RESULTS**

Amplicons of 1.8 kb were produced by primers P1/P7 in direct-PCR (Fig. 1) and ~750 bp was produced by primer pair AV1/AV2 from symptomatic plants 15 plants (Fig. 2). No PCR amplicons were produced from asymptomatic plants. In addition to this direct PCR, the presence of phytoplasma was confirmed by nested PCR by re-amplifying the direct PCR product, which is more sensitive and specific. R16F2n/R2 primers generated an amplicon of 1200 bp in nested PCR (Fig. 3). Therefore, the study suggests association of a phytoplasma with the diseased samples showing little leaf symptom. In the multiplex nested PCR, reaction components and reaction cycling parameters both were standardized. The concentrations of the main ingredients, such as primers,  $MgCl_2$ , dNTPs, Taq DNA polymerase and PCR parameters including annealing temperature

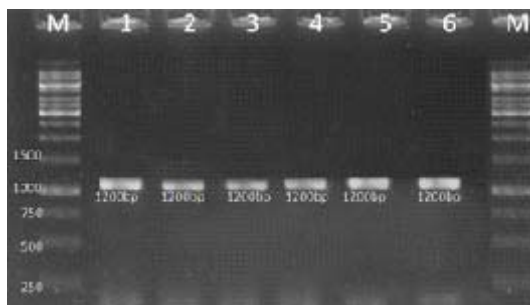
and amplification cycles, were optimized. Expected fragments of ~1800 bp (group 16SrVI, “*Candidatus Phytoplasma trifolii*”) and ~750 bp (Tomato Leaf curl virus) were successfully amplified by this multiplex nested PCR system ensuring simultaneous, sensitive and specific detection of the phytoplasma and the virus (Fig.4). There are very few reports of simultaneous detection of virus and phytoplasma by multiplex PCR (Baranwal *et al.* 2005; Biswas *et al.*, 2013). The resultant 16S rDNA sequence and coat protein gene sequence



**Fig. 1.** PCR amplification of Tomato Leaf Curl virus. M: 1kb ladder. Lane 1, 2, 5: samples showing only mosaic symptom; Lane 6: healthy control



**Fig. 2.** Direct PCR for 16S rDNA amplification of eggplant phytoplasma. M: 1-kb ladder. Lane 1, 2, 5, 7, 9, 10: samples showing yellowing and little leaf symptom

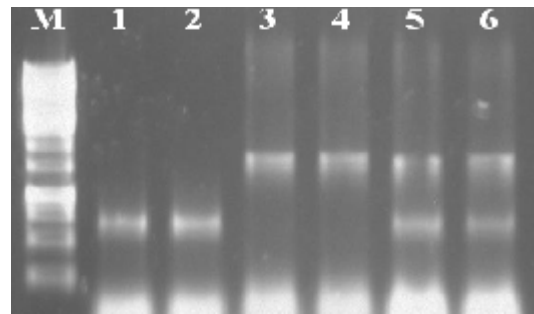


**Fig.3** Nested PCR for 16S rDNA amplification of Eggplant phytoplasma. M: 1-kb ladder. Lane 1, 2, 3, 4, 5, 6: samples showing only little leaf symptom.

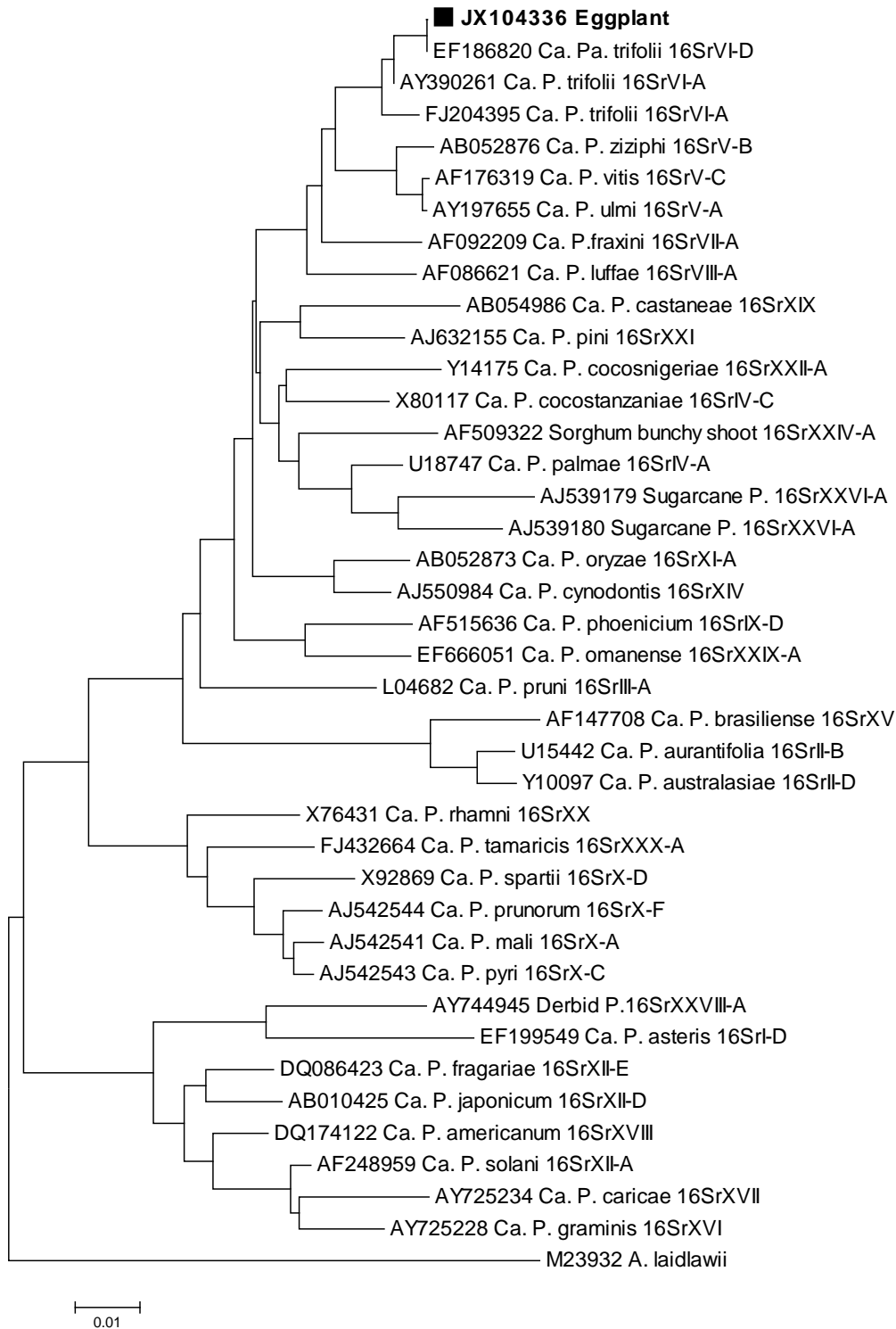
were deposited in GenBank under the accession numbers JX104336 and KC513743. BLAST analysis showed the highest sequence identity (99%) with those of members of group 16SrVI ‘*Candidatus Phytoplasma trifolii*’. Phylogenetic tree constructed based on the analysis of the 16S rDNA sequences from 40 diverse phytoplasmas, showed that the Meerut eggplant phytoplasma closely clustered to subgroup 16SrVI-D (Fig. 5).

The partial coat protein (CP) sequence of the begomovirus detected from symptomatic eggplants (KC513743) showed highest similarity (99%) with that of the begomovirus infecting *Eclipta alba* (DQ339119), the begomovirus infecting *Solanum nigrum* (DQ339123) and begomovirus infecting *Datura metel* (DQ339118) through BLAST analysis. Phylogenetic comparisons showed that the Meerut eggplant begomovirus clustered in a single branch closely related to the whitefly transmitted begomoviruses infecting *Eclipta alba*, *Solanum nigrum* and *Datura metel* in India (Fig.3).

The presence of a 16SrVI phytoplasma in association with whitefly transmitted begomovirus in yellows disease affected eggplants in Meerut, India was verified by PCR assays. Previously phytoplasmas have been found to be associated with eggplant diseases, however, eggplant was known to be immune for begomoviruses and served as a preferred host for whitefly rearing (Czosnek *et al.* 1993). Begomoviruses causing

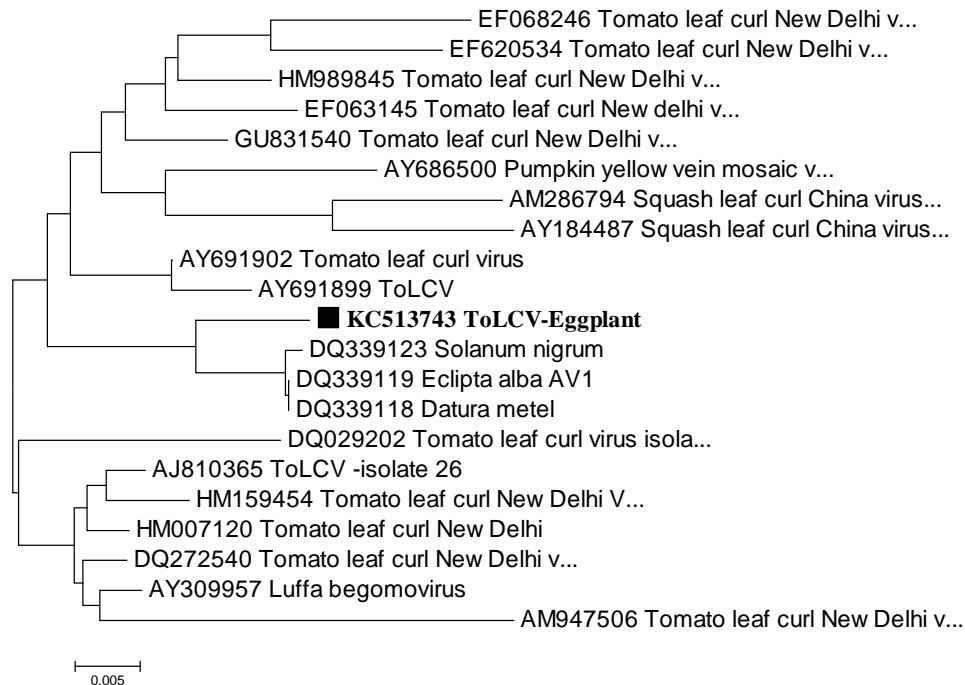


**Fig. 4.** Detection of ToLCV and Phytoplasma in naturally infected eggplant plants using specific primers. M, molecular marker (1-Kb ladder, MBI, Fermentas ), Lane1 and 2: 750 bp amplified product specific to coat protein from primers AV1/AV2, Lane 3-4: PCR amplified product with 16Sr DNA product from primers P1/P7, Lane 5-6: 750 & 1800 bp Multiplex PCR based amplification with Phytoplasma specific P1/P7 and begomovirus universal coat protein primers.



**Fig. 5.** Phylogenetic tree based on the 16S rDNA sequences of eggplant phytoplasma (Accession No. JX104336) with other selected phytoplasmas strains from GenBank. The 0.01 bar indicates one nucleotide change per 100 nucleotides. 'Ca. P' stands for 'Candidatus Phytoplasma





**Fig. 6.** Phylogenetic tree depicting the relationship of various begomoviruses based on alignment of coat protein gene sequences. The sequence under study is highlighted ToLCV-Eggplant; Accession No. KC513743. The viruses used in study are Tomato leaf curl New Delhi Virus (ToLCNDV), Tomat

eggplant yellow mosaic disease (EYMD) have been reported from Thailand (Green *et al.* 2003) and India (Pratap *et al.* 2011). But no reports of phytoplasma-begomovirus mixed infection have been cited in the past and to the best of our knowledge, this is the first evidence of mixed phytoplasma-begomovirus infection in eggplant and report of simultaneous detection of two pathogens by multiplex nested PCR. Phylogenetic analysis on the basis of 16S rDNA phytoplasma sequences showed that Meerut eggplant clustered in the branch embraced by '*Ca. P. trifolii*' and begomovirus isolate identified from infected eggplant clustered with whitefly transmitted begomoviruses infecting different host plants in India.

## DISCUSSION

Mixed infections of phytoplasmas and virus pathogens have been reported in wide range of plant species indicating that both pathogens can infect the same plant host (Arocha *et al.* 2009). These include malformed clovers associated with

rhabdoviruses and phytoplasmas of groups 16SrI, and 16SrX, "*Candidatus Phytoplasma mali*" (Fra'nova' *et al.* 2004); bleeding heart (*Dicentra spectabilis*), in Poland with shoot proliferation, associated with a 16SrI phytoplasma and Tobacco rattle virus (TRV) (Kaminska *et al.* 2005); sugarcane affected by Yellow Leaf Syndrome (YLS) in Mauritius associated with both a luteovirus (SCYLV) and a phytoplasma of group 16SrIII, X-disease (Aljanabi *et al.* 2001; Parmessur *et al.* 2002); a phytoplasma of group 16SrII and Papaya Ring Spot Virus (PRSV) in papaya with Bunchy Top Symptom (BTS) and mixed infection of group 16SrIII-X phytoplasma along with two begomoviruses: Tomato yellow leaf curl virus (TYLCV) and Tomato chino La Paz virus in Pepper (Lebsky *et al.* 2011). The coexistence of 16SrVI phytoplasma and begomovirus in eggplant showing yellow disease symptoms is not completely known and further studies are required to understand the mechanisms by which both are transmitted together to the same plant host, and vector involvement. Detailed analysis of epidemiology and pathogen interaction is required

for such mixed infections and potential role of non-crop species that may act as reservoirs for such plant pathogens. Eco-friendly strategies are needed to be developed for the management of insect vectors that will help in reducing such disease spread to different geographical locations.

#### ACKNOWLEDGEMENTS

We sincerely acknowledge the Vice Chancellor, Sardar Vallabhbhai Patel University of Agriculture and Technology, Meerut-250110, Uttar Pradesh; Council of Science and Technology, Uttar Pradesh and Bioinformatics facility (DBT funded), India, for providing financial support and the facilities to carry out this research work.

#### REFERENCES

- Aljanabi S, Parmessur Y, Motuia Y, Saumtally S and Dookun A., Further evidence of the association of a phytoplasma and a virus with yellow leaf syndrome in sugarcane. *Plant Pathol* 2001; **50**: 628–636.
- Altschul SF, Gish W, Miller W, Myers EW and Lipman DJ., Basic local alignment search tool. *J Mol Biol* 1990; **215**:403-410.
- Arocha Y, Piñol B, Acosta K, Almeida R, Devonshire J, Van De Meene A, Boa E and Lucas J., Detection of phytoplasma and potyvirus pathogens in papaya (*Carica papaya L.*) affected with “Bunchy top symptom” (BTS) in eastern Cuba. *Crop Prot* 2009; **28**(8): 640-646.
- Baranwal V K, Majumder S, Ahlawat Y S and Singh R P., A novel approach for simultaneous detection of *Citrus yellow mosaic virus* and *Citrus greening bacterium* by multiplex polymerase chain reaction. *IJBT* 2005; **4**(4): 528-533
- Bertaccini A and Duduk B., Phytoplasma and phytoplasma diseases: a review of recent research. *Phytopathol Mediterr* 2009; **48**: 355–378.
- Biswas C, Dey P and Satpathy S., A multiplex nested PCR assay for simultaneous detection of *Corchorus golden mosaic virus* and a phytoplasma in white jute (*Corchorus capsularis L.*). *Lett Appl Microbiol.* 2013; **56**(5): 373-8.
- Czosnek H, Kheyr-Pour A, Gronenborn B, Remetz E, Zeidan M, Altman A, Rabinowitch HD, Vidavsky S, Kedar N and Gafni Y., Replication of tomato yellow leaf curl virus (TYLCV) DNA in agroinoculated leaf discs from selected tomato genotypes. *Plant Mol Biol* 1993; **22**: 995-1005.
- Deng S and Hiruki C., Amplification of 16 S rRNA genes from culturable and non-culturable mollicutes. *J Microbiol Methods* 1991; **14**: 53–61.
- Food and Agriculture Organization of the United Nations, FAOSTAT database (FAOSTAT, 2013), available at <http://faostat.fao.org/site/339/default.aspx>
- Fra’nova’ J, Paltrinieri S, Botti S, Simkova’ M and Bertaccini A., Association of phytoplasmas and viruses with malformed clovers. *Folia Microbiol* 2004; **49**(5): 617–624.
- Green SK, Tsai WS, Shih SL, Rezaian MA and Duangsong U., Molecular characterization of a new Begomovirus associated with tomato yellow leaf curl and eggplant yellow mosaic diseases in Thailand. *Plant Dis* 2003; **87**:446-446.
- Gundersen DE and Lee IM., Ultrasensitive detection of phytoplasmas by nested-PCR assays using two universal primer sets. *Phytopathol Mediterr* 1996; **35**: 44-151.
- Kaminska M, Sliwa H and Rudzinska-Langwald A., Shoot proliferation of bleeding heart (*Dicentra spectabilis*) associated with phytoplasma and virus infection. *Phytopathol Pol* 2005; **37**: 33-43.
- Kelly PL, Arocha Y and Dider SZ., First report of 16SrI, ‘*Candidatus* Phytoplasma asteris’ isolate affecting aggpplant and Mikania sp. in Bangladesh. *Plant Pathol* 2009; **58**:789.
- Khan JA, Srivastava P and Singh S K., Efficacy of nested PCR for the detection of phytoplasma causing spike disease of sandal. *Curr Sci* 2004; **86**: 1530–1533.
- Kumar J, Gunapati S, Singh SP, Lalit A, Sharma NC and Tuli R., First report of a ‘*Candidatus* Phytoplasma asteris’ (16SrI group) associated with little leaf disease of *Solanum melongena* (brinjal) in India. *New Dis Rep* 2012; **26**: 21.
- Lebsky V, Hernandez-Gonzalez J, Arguello-Astorga G, Cardenas-Conejo Y and Poghosyan A., Detection of phytoplasmas in mixed infection with begomoviruses: a case study of tomato and pepper in Mexico. *Bull Insectology* 2011; **64**: S55-S56.
- Li Z N, Song J G, Zhang C P, Yu X Q, Wu K K, Wu W J, Wu Y F and Xiang Y., Berberis phyllody is a phytoplasma-associated disease. *Phytoparasitica* 2010; **38**: 99-102.
- Mello APOA, Eckstein B, Flores D, Kreyci PF and Bedendo IP., Identification by computer-simulated RFLP of phytoplasmas associated with eggplant giant calyx representative of two

- subgroups, a lineage of 16SrIII-J and the new subgroup 16SrIII-U. *Int J Syst Evol Microbiol* 2011; **61**: 1454-1461.
20. Okuda S, Prince JP, Davis RE, Dally EL, Lee IM, Mogen B and Kato S., Two groups of phytoplasma from Japan distinguished on the basis of amplification and restriction analysis of 16S rDNA. *Plant Dis* 1997; **81**: 301-305.
  21. Parmessur Y, Aljanabi S, Saumtally S and Dookun-Saumtally A., Sugarcane yellow leaf virus and sugarcane yellows phytoplasma: elimination by tissue culture. *Plant Pathol* 2002; **51**: 561-566.
  22. Pratap D, Kashikar AR and Mukherjee SK., Molecular characterization and infectivity of a *Tomato Leaf Curl New Delhi virus* variant associated with newly emerging yellow mosaic disease of eggplant in India. *J Virol* 2011; **8**: 305.
  23. Rao G P, Mall S, Raj S K and Snehi S K., Phytoplasma diseases affecting various plant species in India. *Acta Phytopathol Entomol Hung* 2011; **46**: 59-99.
  24. Seemuller E, Marcone C, Lauer U, Ragozzino A and Gosch M., Current status of molecular classification of the phytoplasmas. *J Plant Pathol* 1998; **80**: 3-26.
  25. Siddique ABM, Agarwal GK, Alam N, Reddy MK., Electron microscopy and molecular characterization of phytoplasmas associated with little leaf disease of brinjal (*Solanum melongena* L.) and periwinkle (*Catharanthus roseus*) in Bangladesh. *J Phytopathol* 2001; **149**: 237- 44.
  26. Tamura K, Peterson D, Peterson N, Stecher G, Nei M and Kumar S., MEGA5: Molecular Evolutionary Genetics Analysis using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods. *Mol Biol and Evol* 2011; **28**(10):2731-2739.
  27. Thompson JD, Higgins DG and Gibson TJ., CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position specific gap penalties and weight matrix choice. *Nucleic Acids Res* 1994; **22**(22):4673-80.
  28. Wei W, Lee IM, Davis RE, Sau X and Zhao Y., Automated RFLP pattern comparison and similarity coefficient calculation for rapid delineation of new and distinct phytoplasma 16Sr subgroup lineages. *Int J Syst Evol Microbiol* 2008; **58**: 2368-2377.