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Research Article

MOLECULAR CHARACTERIZATION OF PHYTOPLASMA ON TOMATO IN BAHAWALPUR REGION

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Abstract

Tomato (*Solanum lycopersicum* L.) is a most significant and widely planted horticultural crop over the globe, ancestrally belonging to Solanaceae family. Its genetic formation makes it vulnerable to all pathogens (Viruses, Bacteria, Fungi, Nematodes, and Phytoplasmas). After viruses, phytoplasmas are the rising threat to tomato production, as they cause significant yield losses across the globe. During the field visit of tomato growing area in Bahawalpur, tomato leaf samples showing prominent symptoms were collected and nucleic acid extraction was performed to identify the causal agent of the disease. Extracted DNA was further processed using polymerase chain reaction as a molecular characterization technique. After 16S rRNA employing the gene-specific primer pair P1/P7, PCR product of about 1800 bp was amplified, and retrieved sequence was submitted to Genbank (accession Number: OQ651404). The sequence analysis was performed showing the maximum similarity with the Candidatus Phytoplasmas isolated from field bindweed (convolvulus arvensis L) reported from Pakistan (accession Number: MT119155). To the best of our knowledge, this is the first evidence of association of Candidatus Phytoplasmas with leaf shortening disease of tomato from Pakistan. The findings require immediate attention and extensive investigation into their distribution in Pakistan, as well as appropriate interventions.

Keywords: Candidatus Phytoplasmas, GenBank, Phylogenetic analysis, PCR, Sequencing, Tomato.
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Introduction

One of the most significant vegetables in Pakistan as well as the rest of the world is the tomato *(Solanum lycopersicum* L.) Tomato is a member of the Solanaceae family with a short growing season with maximum yield. Tomatoes are a part of a balanced, healthy diet. They are abundant in dietary fiber, carbohydrates, vital amino acids, minerals, and vitamins. Iron, phosphorus, and vitamins B and C are all found in tomatoes. Phytoplasma is a bacterium that lacks a cell wall and is primarily transmitted by leafhoppers. However, it can also be transmitted by seeds and plant propagation materials. It is restricted to the phloem tissues of the plants and has a pleomorphic structure. Leaf shortening, phyllody, flower virescence, and witches' brooms are the prominent symptoms of phytoplasma infection on tomato. They can be turned into juices, sauces, and purees. Regardless of their canning or drying state, tomatoes are processed foods with substantial commercial value. Vitamin A content is higher in yellow tomatoes, whereas lycopene, an antioxidant found in red tomatoes, may help shield against carcinogens. Fresh tomato output amounts to over 159 million tons annually on a global basis. More than 25% of the 159 million metric tons of tomato produced around the world are used for processing, making tomatoes a very popular vegetable (Wang et al., 2023).

Pakistan produced 530 thousand tons of tomatoes in 2011. In Pakistan, the tomato production has a substantial economic worth. Tomato exports from the nation generally averaged 9832 tons per year over the previous 5 years. In 2012, Pakistan exported 5692 tons of tomatoes and made rupees 77 million. Tomato production climbed from 268.8 to 476.8 thousand tones between 2000–2001 and 2012, while the area under tomato crop increased from 28 to 50,000 hectares (Ramirez et al., 2020). Depend on the average of ten-year, the current national tomato output is 10.2 tones/ha, which is relatively low (Paul et al., 2007). High yielding cultivars and more advanced production techniques must be used to achieve a potential yield (MINFAL, 2013).

The introduction of phytoplasmas resistant cultivars is another method of improvement. Eight different "Candidatus Phytoplasma" species have been linked to these infections, which have a variety of regional distribution patterns, in at least 30 different nations throughout the world (Leff et al., 2004). The unique bacteria Phytoplasma, often referred as MLOs, are obligatory microbes of plant phloem tissue as well as of a variety of insect species. Mollicutes' "Candidatus Phytoplasma" family contains phytoplasmas that have separated from gram-positive bacteria (IRPCM, 2004). Various yellows-type plant diseases were thought to be brought on by viruses due to their infectious propagation symptomatology, and insect transmission (Maramorosch, 2008). The possibility that wall-less prokaryote instead of viruses might serve as the disorder's causative agents startled the plant pathology community (Doi, 1967).

The discovery of morphologically wall-less prokaryotic organisms in phloem tubes of different plant species infected with yellows-type disorder was made possible by the identification of a distinct class of bacteria-related pathogens. Because of their ultrastructural and morphological similarities to mycoplasma, these pathogenic microorganisms were initially known as mycoplasma-like organisms. The Mollicutes class of pathogenic prokaryotes includes MLOs and mycoplasma. Unlike to mycoplasma, which are responsible for a variety of illnesses in both plants and animals, the plant-pathogenic MLOs, however, refused all attempt to be cultivated in vitro using cell free culture (Lee and Davis, 1986).

Mysterious status of MLOs among prokaryotes was clarified through the use of molecular technologies, which resulted in the unique, ambiguous classification "phytoplasma" and, subsequently, the classification of new taxon called "Candidatus phytoplasma" (IRPCM, 2004). Phytoplasma-infected plants including tomato, display a wide range of symptoms that point to serious disruptions in the standard balance of growth stimulator. A witch’s broom-like behavior caused by the proliferation of axillary buds, virescence phyllody sterility of flower, aberrant internode elongation, and general stunting are other symptoms (Bertaccini, 2007). Insects from the family of Cicadellidae, Cixidae, Delphacidae, Psyllidae and Derbidae persistently spread these bacteria (Weintraub and Beanland, 2006). Present-day molecular research on Phytoplasma has shed light on their diversity and genetic links, forming the foundation for a number of thorough investigations on Phytoplasma taxonomy and phylogeny (Sumi et al., 2023).

Several studies, in particular 16S rDNA sequence analysis, have demonstrated that Phytoplasma is a cohesive genus-level taxon. According to (Murray & Stackebrandt, 1994) the monophyletic phytoplasmas clades were divided into group and subgroup, many of them are currently regarded as species within the provisional category "Candidatus" for incompletely characterized prokaryotes (1995). To date, a number of preliminary species have been named, and regulations for hypothetical species delineation in the future have been established (IRPCM, 2004). First detail classification scheme of phytoplasmas was rely on restriction fragment particle polymorphism (Lee et al., 1998a, 2000),

Analysis of PCR amplified 16S rRNA, the first thorough phytoplasmas classification scheme was developed offering a trust worthy method for the distinction of a wide range of Phytoplasma. Phytoplasma cannot be grown in laboratory on artificial media with no cells. They are pleomorphic because they do not have cell wall, have such a diameter of less than a millimeter,and icredibly small genomes(675-1600)

One of the most common phytoplasma that cause disease in tomato is Candidatus Phytoplasma spp. When "Candidatus Phytoplasma spp." affects tomato plants, it causes an enormous number of shoots with tiny, light-green foliage and shorter internodes, zero flowers or fruits, and a general decrease that eventually leads to dieback. This method, which categorizes phytoplasma into 19 different groups and higher than 40 subgroups have grown to be the most complete and extensively used approach for categorizing phytoplasmas (Al-Saady et al., 2008).

The study of objectives was to identify and describe the phytoplasmas that were isolated from tomato samples and to do a phylogenetic analysis using the sequences that were taken from the collected samples and the isolates that were accessible in the NCBI from different parts of the world.

Materials and Methods

From Bahawalpur region Yazman, Dera Bakha, Ahmadpur East One hundred and forty (140) symptomatic leaves were randomly collected from each tomato field During month of August to September 2022, various tomato fields in Pakistan were surveyed for the collection of symptomatic leaves of tomato leave exhibiting Phytoplasma-like symptoms including leaf yellowing, leaf curling, thick leaves, small leaves and stunted growth. All Collected samples were brought to laboratory. Asymptomatic plants samples were also collected from a surveyed area One hundred and forty (140) samples were used for the DNA extraction and remaining samples were stored at -80 C for further processing **(Figure 1)**.

Figure 1. (a) Distorted leaves of tomato (b, c) Smaller leaves (d) Shorten internodes (e) Thick leaves (f) Yellowing (g) Curling of leaves (h) Stunted growth.

DNA Isolation

The isolation of the phytoplasmas the DNA of the tomato leaves were extracted using CTAB method and DNA product were analyzed on 1 percent gel in gel electrophoresis apparatus. One gram of tomato leaf was use following protocol described by (Angelini *et al.* 2001).

PCR amplification (16S ribosomal DNA)

To diagnose Phytoplasma disease, one hundred and forty 140 tomato samples were tested with PCR using specific primers. Diseases incidence in percentage was calculated on the basis of results of PCR reaction using **(Table 1)**.

PCR amplifications of obtained sequencing and Phylogenetic analysis

The obtained PCR products of amplification size 1800bp were dispatched to Macrogen, South Korea for one direction Sanger sequencing. The obtained sequences were submitted to NCBI to obtain the accession numbers. The obtained sequences and the sequences retrieved from NCBI were subjected multiple sequence alignment and Phylogenetic analysis. The Phylogenetic analysis was performed using Mega 11 software, using Neighbour Joining Method keeping the bootstrap value at 1000 (Tamura et, al 2021).

Table 1. Primers used for the detection of Phytoplasmas infecting tomato (Smart *et al.*, 1996).

Primer name	Forward primer / Reverse primer	Annealing temperature	Expected size of PCR product
P1/P7	5'-AAGAGTTTGATCCTTGGCTCAGGATT-3' 5'-CGTCCTTCATCGGCTCTT-3'	35	1800
R16F1/R16R0	5'-AAGACGAGGATAACAGTTGG-3' 5'-GGATACCTTGTTACGACTTAACCCC-3'	35	1354
R16mF2/R16mR1	5'-CATGCAAGTCAACGGA-3' 5'-CTTAACCCCAATCATCGAC-3'	35	1435
R16F2/R16R2	5'-ACGACTGCTGCTAAGACTGG-3' 5'-TGACGGGCGGTGTGTACAAACCCCG-3'	35	1248
P1/Tint	5'-AAGAGTTTGATCCTGGCTCAGGATT-3' 5'-TCAGGCGTGTGCTCTAACCAG-3'	35	1600

Results

Disease incidence on based PCR amplification

Bahawalpur, Yazman, Dera Bakha, and Ahmadpur East had illness incidence rates of 74.28%, 74.28%, 62.86%, and 71.43%, in that order. In Bahawalpur, the overall disease incidence was 71%. (**Table 2).**

Sequence and Phylogenetic analysis of Candidatus Phytoplasmas spp on tomato

Partial sequence of the amplified product from diseased tomato was confirmed to be Candidatus Phytoplasma spp when it was subjected to BLAST analysis. The obtained Phytoplasma sequence was submitted to NCBI with accession number (OQ651404) and had the greatest homology to another isolate reported from bindweed in Pakistan (MT119155) (Nabi et al., 2015). The obtained results demonstrated a higher degree of similarity among all nucleotide sequences, varying from 97 to 100%. A range of 95% to 100% was seen in the similarity rates of the isolates found in GenBank that represented India and other nations. Pakistani isolates were found to be less identical to those from Egypt (OP642367, OP723297, OP723296), India (ON975015, ON975012, ON975019, ON975020, OP032751, OQ549913, ON975009), Iran (OQ448205) and Sri Lanka (OQ451841). The generated Phylogenetic tree of Candidatus Phytoplasma spp. showed that the resemblance to other phytoplasmas found all over the world. Phylogenetic analysis revealed that the isolates shared low genetic diversity as they were segregated in two clades i.e., A and B. The Clade A contained all the isolates reported from different countries and clade B was an independent group of isolates from Pakistan, one reported from bindweed and other newly characterized isolate from tomato **(Figure 2)**.

Table 2. Disease Incidence of Phytoplasma in Bahawalpur region

Location	No. of tested samples	No. of sample with yellowing of leaves	No. of sample with curling of leaves	No. of sample with stunted growth	No. of sample with thickness of leaves	Disease incidence percentage (%)
Bahawalpur	**35** 26 positive 9 negative	**09** 6 positive 3 negative	**10** 8 positive 2 negative	**09** 7 positive 2 negative	**07** 5 positive 2 negative	**74.28%**
Yazman	**35** 26 positive 9 negative	**08** 6 positive 2 negative	**09** 7 positive 2 negative	**11** 9 positive 2 negative	**07** 4 positive 3 negative	**74.28%**
Dera Bakha	**35** 22 positive 13 negative	**15** 11 positive 4 negative	**08** 4 positive 4 negative	**08** 5 positive 3 negative	**04** 3 positive 1 negative	**62.86%**
Ahmadpur East	**35** 25 positive 10 negative	**08** 6 positive 2 negative	**09** 7 positive 2 negative	**09** 5 positive 4 negative	**09** 7 positive 2 negative	**71.43%**
Total	**140** 99positive 41 negative	**40** 29 positive 11 negative	**34** 26 positive 8 negative	**37** 26 positive 11 negative	**27** 19 positive 8 negative	**71%**

***Positive** = leaves infected with phytoplasmas. ***Negative**= healthy leaves.

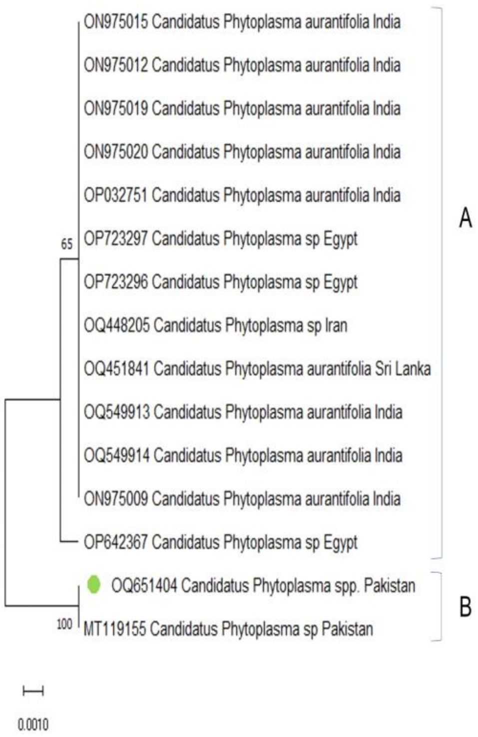

Figure 2. Phylogenetic Tree Showing the Relationship between different isolate of *Candidatus Phytoplasmas spp* isolated from different host in different regions of the world

Discussion

Tomato Phytoplasma pathogens have previously been documented in a number of nations, such as Poland (Krawczyk *et al.*, 2010), China (Xu *et al.*, 2013), Mexico (Tapia-Tussell *et al.*, 2012), Egypt (EL-Banna *et al.*, 2007), Brazil (Mello *et al.*, 2006), Jordan (Anfoka *et al.*, 2003), Turkey (Ozdemir *et al.*, Del Serrone *et al.*, 2001). In recent years, emerging Phytoplasma diseases have increasingly become important in Pakistan, due to their serious impact on economically important crops, therefore, precise diagnosis, including pathogen identification is essential for disease control and preventing further infection spread. In the current study, visual inspection in Bahawalpur region (Bahawalpur, Yazman, Dera Bakha, and Ahmadpur East) particularly dedicated to tomato production. Applicant as a member of the Mollicutes class, Phytoplasma spp. appear to be wall less, non-cultivable, non-helical, and phloem-restricted prokaryotes with in the scientific Phytoplasma family 16Sr XII. It is the most significant tomato Phytoplasma responsible for the Witches' broom infection (Wei et al., 2016). Tomato plants naturally infected with Phytoplasma *Candidatus Phytoplasmas spp.* showed a range of symptoms including leaf yellowing, leaf curling, thick leaves, small leaves and stunted growth and witches'-broom as observed by many other researchers (Du *et al.* 2013; Xu *et al.*,2013). Symptoms observed on tomato plant includes excessive greening, witches'- broom reduced plant height and modification of floral structures into leaf like structures that lead to phyllody as previously reported by (Janke *et al.,* 2007). In addition to tomato, it also damages other domesticated, wild, herbaceous, and timber plants, including grapevine, where it generates infection (Mall et al., 2023). These results indicate the presence of Phytoplasma in the phloem of symptomatic plants as previously reported by (Akhtar *et al.,* 2016b) and many others. Six species groups (16SrI, II, III, V, VI, and XII) of phytoplasmas with phylogenetically distinct strains have been reported to infect tomato plants all over the world (Asghari Tazehk *et al.,* 2017). Several weeds are reported to be the natural alternative hosts of important phytoplasmas by spreading these pathogens to cause serious diseases in important commercial crops (Nabi *et al.,* 2015). Weed plants could play a key role in the epidemiology of the diseases caused by Phytoplasma since they influence the population density of its vectors and can also act as a potential inoculums source (Pasquini *et al.,* 2007). While bactericides, such as antibiotics, against Phytoplasma are ineffective, the introduction of more resilient tomato cultivars may be an alternate method of control. Pesticide-based prevention of insect vectors is a workable strategy to prevent the further propagation of Phytoplasma. Tetracycline treatment for sick plants is another strategy for managing Phytoplasma infections (Bertaccini, 2021). This is not a long-term preventative measure, though, as these plants are vulnerable to reinfections when exposed to insect vectors again. Moreover, antibiotics are excessively expensive and their use is restricted in many nations. A further indication that gibberellic acid on diseased tomato plants caused symptom recovery was the absence of flowers and fruits in any of the tomato cultivars treated with antibiotics (Kang *et al*., 2012). Additionally, an increasing recovery rate was reported with gibberellic acid treatment followed by ledermycin. For the management of plant diseases, a number of bio-agents are also used. Arbuscular mycorrhizal (AM) fungus treatment of tomato plants infected with "stolbur" phytoplasma resulted in reduced symptom manifestation and Phytoplasma cell degeneration (Kumari *et al*., 2019). Studying the effects of various molecules, including *biophenicol, clarithromycin, enteromycelin, paraxin, lycercelin, roscillin, campicillin, oxytetracycline, chlorotetracycline*, and rose clove eucalyptus oils, on Phytoplasma-infected brinjal (*solanum melon Gena*) cultivars revealed that they were largely ineffective because they failed to significantly control the disease (Marcone *et al*., 2019). Management techniques include rouging of sick plants, adjusting the sowing date, using clean propagation material, rotating with non-host crops, and removing weeds in conjunction with vector control are beneficial. The pickup of phytoplasmas is different from that of fungus or bacteria because they are dependent on a living host for survival, making the use of a single chemical impractical. Hence, an integrative system that incorporates elements of cultural, physical, microbiological, defensive, and medicinal applications may be the most practical and sustainable choice. These earlier findings stated that the reported Phytoplasma from 16SrRNA groups I, II, III, V, VI, and XII cause the illnesses. The results of this study demonstrated a correlation between the symptoms of yellowing, chlorotic, and deformity in tomato plants and the Phytoplasma infection in samples collected from the Bahawalpur district of Pakistan. Tomato sample phytoplasmas showed similar symptoms; however, in this study, 16SrII was found.

Conclusion

In the present study the infected tomato samples showing prominent symptoms leaf yellowing, leaf curling, thick leaves, small leaves and stunted growth were molecularly characterized and sequenced, the retrieved sequence was submitted to gene bank (OQ651404).The phylogenetic analysis reveals the maximum similarity with (MT119155) based on the identification of causal agent of these infected tomato plants, management practices can be ruled out/employed to overcome this utmost arising threat of tomato crop. Developing effective resistance breeding program can help to mitigate the economic impacts of this threat. This study contributes significantly to understand phytoplasma disease in tomato crops, informing evedance based management strategies to safeguard Pakistan's agriculture sector.

Authors' contributions

Conceived and designed the experiments: (Details of Teacher and Authors)

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