

Detection of *Spiroplasma citri* causing citrus stubborn disease in Sargodha, Pakistan

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Abstract

Citrus fruit is considering very nutritious fruit. Citrus trees are affected by the several viruses, bacteria, fungi and virus-like diseases. In latest years, citrus trees are seriously affected by a prokaryotic pathogen *Spiroplasma citri* that cause stubborn disease. On affected trees, fruits become small, crooked and seeds are aborted. The present study was planned to diagnose the pathogen through Sp4 media. *Spiroplasma citri* was isolated from infected leaves and columella part of fruit. Culture inoculated cultured media were incubated at 30°C. After 7-14 days of incubated media color was changed from red to yellow which showed the initial presence of *S. citri*. Typically, fried egg shape colony was observed on solid media. Molecular detection of *Spiroplasma citri*, DNA was extracted from leaves sample. Spiroplasma specific Spiraline-f and spiraline-r primer pair was used in to amplify spiraline gene with 675bp product. Pathogenicity test was performed. Upon successful grafting, symptoms were observed that confirmed the presence of disease. Moreover, detection of citrus stubborn leads to the first-time identification of *S. citri* on media, biologically and molecularly in the citrus orchards of Sargodha.

Keywords: citrus stubborn, isolation, fried egg shape colonies, DNA extraction, grafting

1. Introduction

Citrus belongs to one of largest group of trees and shrubs. Citrus fits to Sub-family *Aurantioideae*, family *Rutaceae* and genus *Citrus* L. It originated from southeast Asia although some research points to Australia, New Caledonia and New Guinea (Khan *et al.*, 2016) [13]. Citrus being nutritious and delicious fruit is mostly grown in tropical and subtropical climate. Citrus and citrus products play crucial role in growth and development by providing minerals and nutrients i.e. carbohydrates, vitamin B and C, calcium, potassium, thiamine, magnesium, folate etc., (Economos and Clay, 1999; Sanofer *et al.*, 2014) [6, 25]. Pakistan is 13th largest citrus producing country since 1995-96, fresh citrus export is increasing (knoema, 2019) [14]. Citrus is being affected by both biotic (viruses, bacteria, fungi and virus like diseases) and abiotic factors. Among the biotic factors, prokaryotes i.e. bacteria (*Xanthomonas* spp.) and wall less bacteria (*Spiroplasma citri*) are significantly causing losses in terms of citrus production (Bové and Garnier, 2002; Mubeen *et al.*, 2015a, b) [4]. *S. citri*, causing citrus stubborn disease, belongs to family *Spiroplasmataceae*, genus *Spiroplasma* and class *Mollicutes*. A type species of genus *Spiroplasma*, *S. citri* is mainly found in phloem sieve tubes and phloem sap feeding insects serve as vector (Gumpf *et al.*, 1981; Bové *et al.*, 2003) [9, 4], it make *Spiroplasma* the only mollicutes showing motility with helical morphology. *Spiroplasma citri* are transovarially transmitted. Obligation of cholesterol for growth, complete resistance to penicillin, low guanine and cytosine content of cellular

Deoxyribonucleic acid (DNA), small genome size, and numerous cultural properties (André *et al.*, 2005). Ahmad *et al.*, (2015) reported the presence of *S. citri* in Pakistan citrus producing areas. The disease stubborn is named after persistence of original characters of the tree. The major symptoms include little leaf, mottled or chlorotic, abnormally upright, cupped ultimately whole plant is more or less stunted. Sometimes shoots are abnormally branched with axillary bud gives appearance of witch's broom. The fruit has de-shaped with loss in production (Manna *et al.*, 2013) [16]. In field, *S. citri* is primarily transmitted by leafhoppers while long distance transmission is through infected budwood. In field condition the diagnosis of stubborn disease is difficult due to symptom resemblance with nutritional deficiency (Mello *et al.*, 2010) [17]. Keeping in view all these facts, the aim of present study to identification of *S. citri* based on symptoms, isolation and molecular detection, vegetative transmission of *Spiroplasma citri* and updating the current status of *S. citri* infecting citrus growing areas of Pakistan.

2. Materials and Methods

2.1 Collection of specimens

Infected leaves, fruits and young stems were collected from citrus orchards located in field of Sargodha on basis of symptoms (Fig.1). Samples were brought to the Plant Pathology Laboratory, College of Agriculture, University of Sargodha. After surface sterilization samples were stored at -20°C for further studies.



Fig 1: Infected leaves were cupped, thick, mottle or had chlorotic pattern and often had vertical appearance showed fruits were smaller in size and acorn-shaped and Fruits gave hard appearance like stone.

2.2 Culturing and Isolation of *S. citri* on sp4 medium

The samples were diced in 5-mL of sp4 medium (Igwegb, 1978) and left for 5 min. (Garnier and Bove, 1981) [3]. Medium was then passed through 0.45µm filter membrane. The 1-mL of filtrate was transferred to 10-mL of sp4 medium followed by shaking incubation at 32°C. After 5 and 14 days, culture were examined for growth. The 0.1-mL of medium from culture tubes with changed colored was further transferred to solid medium.

2.3 Purification and preservation of pathogen

Pure culture was obtained by inoculating single colony on petri plates and pure culture were preserved at 4°C on sp4 medium. Cultures were maintained by giving passage after 15 days on new medium for continuity of its viability.

2.4 DNA extraction and PCR amplification

The fresh infected leaves and fruits samples were used for DNA extraction. Cetyltrimethylammonium bromide method was used for DNA extraction (Doyle and Doyle, 1987) [5]. Suspected *S. citri* was confirmed by polymerase chain reaction. Extracted DNA was used as template. Spiraline-f (5'GTCGGAACAACATCAGTGGT³) /spiraline-r (5'TGCTTTTGGTGGTGCTAATG³) primers (spiraline gene had 675bp product) were used for amplification (Yokomi *et al.*, 2008) [17].

2.5 Transmission of stubborn disease through budding

Two years old citrus plants of different varieties (Cara caranavel, Red blood, Feuterell's early, Salustiyana, seed less lemon, Kinnow, Tarocco, Shamber, Murcott, Desi sweet lime, Musambi, Succari) were used for transmission. Four plants of each variety and a control were used. Infected Single bud was used from desired scion for budding. Symptoms were observed after 6-7 weeks after budding.

3. Results

3.1 Isolation of *Spiroplasma citri*

The pathogen was cultured on sp4 medium. Color of sp4 media was pale red. After 7-14 days of inoculation color was changed from red to yellow which showed the presence of

S. citri in the media. A fried egg shape colony of pathogen was observed on solid media (Fig.2. A, B, C, D).

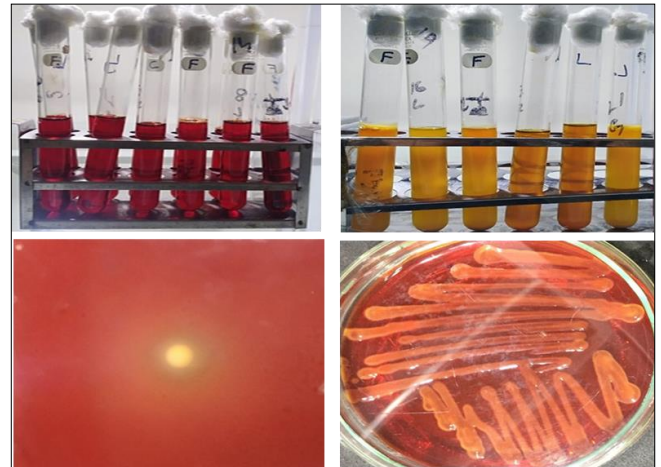


Fig 2: Characteristic of the *Spiroplasma citri* when inoculated in both both and solid medium. Inoculated tubes (a), color of inoculated tubes changed after 7-14 days of incubation (b), egg shape colony of *Spiroplasma citri* (c) and streaking of *Spiroplasma citri* on solid media (d).

3.2 Transmission through budding

The citrus varieties produced symptoms after 7 weeks. Leaves were thicker, cupped shaped. and had mottle or chlorotic pattern (Fig. 3).



Fig 3: Major symptoms caused by *Spiroplasma citri*

3.3 Molecular detection of pathogen

Gel electrophoresis of PCR product showed the presence of *Spiroplasma citri* in samples using primers spiraline-f/spiraline-r which amplified the gene at 675 bp fragment (Fig. 4). The 100 bp DNA ladder was used.

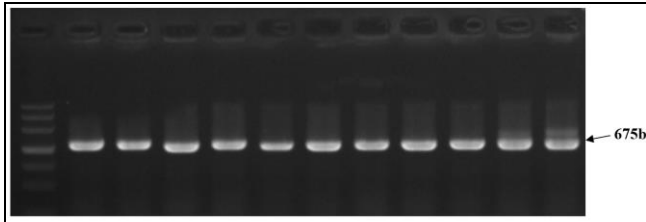


Fig 4: Gel electrophoresis

4. Discussion

Spiroplasma citri disease is one of the destructive diseases of citrus. It causes enormous losses over the world. The pathogen was isolated from infected samples and detected by polymerase chain reaction. Samples were collected from infected field of Sargodha. Trees that were affected by stubborn disease habitually produced less fruit, smaller in size, and acorn-shaped. Infected tree had stunted growth and gave witches broom appearance. Leaves were smaller, cupped and often had vertical appearance. Leaves were thick, mottle or had chlorotic pattern. Fruits were smaller in size, acorn-shaped and had hard appearance like stone. Seeds were also aborted. Infected trees had stunted growth and produced small size fruits (Shi *et al.*, 2014) [26]. Symptoms related to tree size and fruit yield are likely related to the fact that *S. citri*, a phloem resident, requires carbohydrates and sterols from its plant host (Wei *et al.*, 2006) [27]. While living in plants, *Spiroplasma* competes with their hosts for these energy sources, causing depletion of some sugars and hormones and accumulation of others. The causal agent was isolated from young leaves of suspected plants. Results of various tests conducted led to the identification and cultivation of the causal organism, *Spiroplasma citri* (Mollicutes, *Spiroplasmataceae*) i.e. change the color of cultivated medium from red to yellow indicated the presence of *Spiroplasma* in the cultivated medium. The change in color was attributed to the increase in acid production resulted from the culture growth. Reduction in the media pH caused the pH indicator, the phenol red to change to yellow. Similar results were previously demonstrated when *Spiroplasma* were cultured similar medium (Lee and Davis, 1984; Omar; 2006) [15, 23]. This presence of *S. citri* was further confirmed by the appearance of typical fried-egg shape colonies on sp4 medium containing 0.8%. Nobel agar inoculated with the isolates. The colonies were very small in size, appeared as pinheads and clear in color. These colonies were circular in form with a dark centre surrounded by a lighter color, when examined under light microscopy. The observations were in line with those obtained by several workers (Saglio *et al.*, 1973) [24].

The fried egg colonies developed because the helical mollicute, *S. citri* when growing on low-agar medium, would form fuzzy colonies with occasional surrounding satellite colonies due to the ability of the *Spiroplasmal* cells to move and penetrate through the agar matrix (Jacob *et al.*, 1997) [12]. Sp4 media was also used by Nejat *et al.*, (2011) [20] described the same results about the media. After inoculation of one-week media color was changed from red to yellow which was similar to our results. Color of media was changed due to increase the production of acid. As the production of acid increased, the pH of cultivated media was changed. Phenol red (pH indicator) changed to yellow. Our results were also collaborated by El Fatah *et al.*, (2016)

[7]. They described that the growth of *Spiroplasma citri* in cultivated media was followed by the changing of phenol red color into yellow. Phenol red was the pH indicator. When acid production was high in cultivated media the pH of media was changed and color of phenol red was changed into yellow. On solid media, fried egg shape colony of *Spiroplasma citri* was observed. PPLO agar was added to make the solid media. Colony was small in size and in circular shape. At the center, colony had dark color and surrounded by light color. Our results were also supported by Fudl-Allah *et al.*, (1973) [8]. They illustrated that on agar media *Spiroplasma citri* formed fried egg-shaped colony. Which was darker and dense at the center surrounded by light color Colony had circular zone. After about 15 days, colony had 0.1-0.2 mm diameter.

Our results were also collaborated with El Fatah *et al.*, (2016) [7]. They demonstrated that by the addition of 0.8% agar in the medium *Spiroplasma citri* formed fried egg shape colony. Colony was in circular form and had dark color at the center surrounded by light color. These symptoms were like those described by Shi *et al.*, 2014 [26]. They described affected trees had stunted growth. Leaves were smaller in size and had mottle or chlorotic pattern. Fruits were abnormally matured and had aborted seeds. Fruits were dropped while small. Across the top, affected trees were often flattened. Symptoms of our infected samples were also supported by Manna *et al.*, (2013) [16]. They also demonstrated that leaves were mottled. Affected trees were with stunted growth and had shortened leaf internodes. Affected trees had immature and lopsided fruits. Pathogen was isolated from the infected young leaves of citrus. Various tests were conducted for the identification of *Spiroplasma citri*. Cultivated media changed the color from pale red to yellow showed the presence of *Spiroplasma citri*. Similar symptoms were also illustrated by Nejat *et al.*, (2011) [21] described that leaves were smaller in size and had chlorotic pattern. Fruits were acorn shaped and small. Plants were dying and stunted. Seeds were aborted, and acorn shaped. *Spiroplasma citri* was detected by using the polymerase chain reaction. Pathogen DNA was extracted from the leaves and columella part of collected plant samples. Extracted DNA was used as template. Spiraline-f / spiraline-r primers were used in amplification spiraline gene had ~675bp product.

For detection same primers were used by Yakomi *et al.*, (2008). They described that DNA was extracted from infected samples. They used spiraline-f/ spiraline-r primers which gave expected 675bp molecular product. Manna *et al.*, (2013) [16] used P89-f/ P89-r and spiraline-f/ spiraline-r primers for the detection of *Spiroplasma citri*. They described that spiraline primers gave best result when processed with samples that were collected in summer. P89 primers were specific and efficient for the detection of *Spiroplasma citri* of Egyptian isolates. Our same primers were also used by El-Fatah *et al.*, (2016) [7]. They extracted DNA from the infected samples by using Dellaporta extraction method. They used spiraline-f/ spiraline-r primers for the amplification of spiraline gene of 675 bp product. Transmission of *Spiroplasma citri* was detected by using biological indexing on indicator plants. Symptoms of stubborn disease was observed after 6-7 weeks of inoculation on indicator plants which included cupped shaped leaves, chlorosis or mottling pattern. Our same results were also collaborated with the results of Manna *et*

al., (2013)^[16]. They were used biological indexing to check the transmission of *Spiroplasma citri*. After 4-5 weeks of post inoculation they observed the symptoms of stubborn disease. They observed leaves were smaller in size, cupped shape and had chlorotic or mottling pattern. Graft transmission was also used by Nejat *et al.*, (2011)^[20] they used periwinkle plants for graft transmission. After 4-5 weeks of transmission, periwinkle plants showed symptoms of stubborn disease caused by *Spiroplasma citri*. They observed cupped shaped leaves that had chlorotic pattern. Leaves were smaller in size. Their results were also collaborated with our results. Our results were also collaborated with results described by Arif *et al.*, (2005)^[2]. They performed biological indexing to check the transmission of *Spiroplasma citri*. After 3-4 weeks of grafting similar symptoms were observed on indicator plants. Leaves were chlorotic and smaller in size. They also observed curling of leaves. Citrus stubborn is considered an emerging disease in Pakistan. There is scanty of data regarding this disease. It is responsible along with other pathogens for causing decline in citrus orchards. It is trivial to isolate and detect the citrus stubborn pathogen firstly to manage the disease efficiently and prescribe its control measures.

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