



Full Length Research Article

Molecular characterization of a phytoplasma associated with mango malformation and witches broom disease in Saudi Arabia

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ABSTRACT

Background: Many diseases have been reported on mango trees; however, recently, another important pathogen, phytoplasma, has emerged as a severe threat to mango. Phytoplasma belongs to the class Mollicutes.

Methods: Different symptoms, such as malformation and witches' broom are associated with phytoplasma infection were found in mango orchards in Al-Baha region of Saudi Arabia in 2022. Mango phytoplasmas were tested with degenerate phytoplasma primer (P1/P7) and nested primers R16F2n/R16R2 using polymerase chain reaction (PCR). For further confirmation, a PCR reaction was performed using universal phytoplasma primers fU5 and rU3.

Results: Nested PCR with two universal primer pairs, R16F2n/ R16R2, successfully amplified 1.2 Kb and 880 bp with fU5 and rU3 nested primers. The results confirmed the presence of phytoplasma in all collected samples. The obtained nucleotide sequences of three positive samples shared 100% identity among themselves and shared 98.9% to 100% with the 16SrIX strains available in the NCBI database. These sequences were submitted in GenBank under the following accession numbers: OQ914356 (Mango-SA-1), OQ914357 (Mango-SA-2), and OQ914358 (Mango-SA-3).

Conclusion: These results indicate that the Southwest regions of the kingdom have been affected by phytoplasma-induced disease. Also, the present study plays a significant role in detecting and managing this disease in other mango-growing areas of Saudi Arabia. To the best of our knowledge, this is the first report of phytoplasmas in mango from Saudi Arabia.

INTRODUCTION

Mangifera indica, known as the king of fruits, belongs to the family *Anacardiaceae*, which plays an economically crucial role worldwide [1]. Despite mangoes being grown in more than 120 countries, just 15 of those nations account for more than 1% of global production. Over 60% of the world's mango crop is produced in Mexico, China, India, Thailand, and Indonesia [1]. Many pathogens severely damaged the mango tree; the most common include bacteria, fungi, and viruses, which caused significant economic losses [2]. Through mango growing in multiple regions covering over 6,966 hectares, the Kingdom of Saudi Arabia has attained a 68% self-sufficiency rate in mango production, surpassing 89,500 tons annually (2024), according to the Ministry of Environment, Water, and Agriculture, whereas the increasing incidence of disease may pose a potential threat to mango production (<https://www.spa.gov.sa/en/N2141990>).

Phytoplasma is one of the main pathogens of mango trees and belongs to the class Mollicutes. Different types of symptoms appeared due to the phytoplasma infection, and it has been reported that 700 plant species are infected with phytoplasma all over the world [3]. Before 1967, when a team of Japanese scientists employed electron microscopy to find bacteria that resembled animal mycoplasmas found in phloem tubes of diseased plants, many yellows-type diseases, including aster yellows and paulownia witches' broom, were thought to be caused by viruses [4]. The genus *Candidatus Phytoplasma* was designated when the innocuous name "phytoplasma" was approved [5]. Phytoplasma-infected plants frequently exhibit severe imbalances in growth regulators. Atypical internode elongation, phyllody of flowers and virescence, infertility, decline in apical dominance leading to the multiplication of axillary buds with the production of witches' broom, and generalized stunting are among the symptoms [6-8]. For an initial indication that phytoplasma may be involved in a disease, distinctive symptomatology is highly helpful. Only indirect biological evidence, such as electron microscope examination, amplification of a particular DNA, and symptom reduction using tetracycline, supports the existence of phytoplasmas as plant diseases, even after fifty years since their discovery [9]. The primary methods for confirming the link between phytoplasma and plant disease are insect and dodder transmission. During the field survey, phytoplasma-like symptoms were observed in Bisha, Al-Baha, Saudi Arabia. The aims of this study were to identify and describe phytoplasma-like symptoms associated with mango trees in the Al-Baha region of Saudi Arabia and to examine their correlation with other phytoplasma isolates worldwide.

METHODS

Source of Samples and Sample Collection:

A field visit was conducted in 2022, and 25 asymptomatic and symptomatic plant samples exhibiting witches'-broom-like symptoms were collected from mango trees growing in Bisha, Al-Baha Region, Saudi Arabia (Fig. 1). These samples were tested for the detection of phytoplasma using universal and nested primers.

Genomic DNA isolation and PCR amplification

DNA was isolated from mango samples according to the manufacturer's protocol using QIAGEN DNA Purification Mini-Kit. P1/P7 direct phytoplasma universal primer pairs were used to amplify the 16S rRNA phytoplasma gene linked to mango witches' broom. The DNA was amplified using 35 cycles, 30 sec at 94°C, 1 min at 55°C, 1 min at 72°C, and a final 10-min extension at 72°C [10-14]. Using the specific primer pair R16F2n and R16R2, 2 µL of the first-round PCR product was utilized as a template in a final volume of 25 µL for the nested reaction. A total of thirty-five cycles were performed, 1 min of denaturation (two minutes for the first cycle) at 94°C, 2 min at 50°C, and 5 min of extension (10 minutes for the last cycle) at 72°C [15-17].

For confirmation, universal phytoplasma PCR kit, Cat No. 08009C/100 from Loewe Biochemica GmbH (Germany) with primer pairs fU5: (5'-cggcaatggaggaaact-3') and rU3: (5'-tggtacaaagagtagctgaa-3') [18] were used and amplified phytoplasma 16S rDNA gene (880 bp) on the phytoplasma 16S rDNA gene. Reaction was performed according to the manufacturer's protocol.

PCR products (5 µL) were analyzed by agarose gel electrophoresis (1%) in 1XTBE Buffer pH 8.3 (89 mM Tris-Borate and 2mM EDTA; Invitrogen), stained with 5 µL Acridine orange, and visualized with UV trans illuminator (ULTRA LUM, Ultraviolet Transilluminator, Inc, Carson,

California, 90746). A healthy mango sample was used as a negative control [19]. The 50 bp HyperLadder™ II DNA marker (Bioline Ltd, USA) was used to compare our results.

Nucleotide sequencing and phylogenetic tree analysis:

The three amplified PCR products with fU5 and rU3 primers were excised and purified using the Qia-quick PCR Purification Kit from Qiagen. These samples were sent to Macrogen Inc., Seoul, Korea, for two-directional sequencing. The obtained sequences were analyzed using the BLAST program in the NCBI database [20].

DNASTAR software was used to align the 16S rRNA gene sequences from PCR, as well as those from reference phytoplasmas that were obtained from GenBank [21]. A phylogenetic tree was constructed from ClustalW- aligned sequences in MEGA- X using the Maximum- Likelihood approach and 1000 bootstrap replications, as described by Tamura et al. (2004). Different phytoplasma strains retrieved from NCBI were used in the comparison (Table 1).

RESULTS

Field Observation

A field visit was conducted in 2022 at a mango orchard growing under field conditions in the Al-Baha region of Saudi Arabia. The trees showed typical symptoms of phytoplasma witches' broom, including upward-rolling leaves, yellowing, discoloration, and shortened internodes (Fig. 1).

PCR Reaction

Nested PCR with two universal primer pairs (R16F2n/R16R2 and P1/p7) successfully amplified six samples with the expected size of 1.2 Kb in the first-round whereas, there was no amplification seen with the rest of non- symptomatic samples (Fig. 2). These six positive samples were further analyzed by Universal phytoplasma kit from Loewe Biochemica GmbH, (Germany) with fU5 and rU3 as a nested primer to amplify 880 bp comparing them with 50bp HyperLadder™ II DNA marker. The obtained results showed the confirmation of phytoplasma in all these positive samples (Fig. 3). These results indicate that regions of the kingdom in the Southwest have been affected by phytoplasma-induced disease.

Nucleotide sequencing and Phylogenetic analysis

After the sequence analysis of three PCR products obtained with fU5 and rU3 nested primer from mango showed their similarity with phyllody phytoplasma (16SrIX) and were uploaded to NCBI database under the following accession numbers; OQ914356 (Mango- SA-1), OQ914357 (Mango- SA-2), and OQ914358 (Mango- SA-3). The phylogenetic tree of three Saudi Arabian phytoplasma isolates showed four different clades with other isolates posted in the GenBank (Fig. 4).

According to the phylogenetic tree and pairwise nucleotide identity analysis, Saudi Arabian phytoplasma isolates grouped together with 16SrIX (pigeon pea witches' broom) five Iranian isolates isolated from Lettuce, *Lactuca sativa*, Sainfoin, *Onobrychis viciifolia*, Beggarticks, *Bidens alba*, Large Flower Tickseed, *Coreopsis grandiflora*, and Lettuc, *Lactuca serriola*, one published Saudi isolate isolated from chicory, *Cichorium intybus* and two Indian isolates isolated from Wax gourd, *Benincasa hispida* and cowpea, *Vigna unguiculata*. The nucleotide percentage identity indicated that, the three Saudi Arabian phytoplasma isolates of mango shared 100% similarity among themselves and 99.8-100% with the other isolates available in the NCBI database. However, these sequences shared the highest similarity (99.7-99.9%) with five isolates from Italy isolated from *Gladiolus* spp. (OL636385), *Osbornellus horvathi* (KU896990); *Knautia*, *Knautia arvensis* (Y18052), *Picris*, *Picris echioides* (KF932284) and periwinkle, *Catharanthus roseus* (JN791266), four isolates from Lebanon isolated from apple, *Malus domestica* (KP851766), Lettuce, *Lactuca serriola* (AF515638) and *Osyris*, *Osyris alba* (KP851768), wild mustard, (*Sinapis arvensis* (KP851772), five isolates from Turkey isolated from Leafhopper, *Neoliturus haematoceps* (KY417154), eggplant, *Solanum melongena* (MK876848), Lettuce, *Lactuca sativa* (ON454285), stinking hawksbeard, *Crepis foetida* (OM681509) and sesame, *Sesamum indicum* (KC139791), ten isolates from Iran isolated from *Conocarpus erectus* (MT712211), grapevine,

Vitis vinifera (KX011516), *Sophora*, *Sophora alopecuroides* (KX172135 and KJ001834), *Robinia pseudoacacia*, *black locust* (KX553992), *Sophora*, *Sophora alopecuroides* (KF932283), sesame, *Sesamum indicum* (JX464672), periwinkle, *Catharanthus roseus* (ON386808), peach, *Prunus persica* (KF932284), tomato, *Solanum lycopersicum* (JF508510), two isolates from India isolated from *Brassica*, *Brassica rapa* cv. Toria (HM988986 and HM559245), and one isolate from Pakistan isolated from *Brassica campestris* (KU892213).

Figures

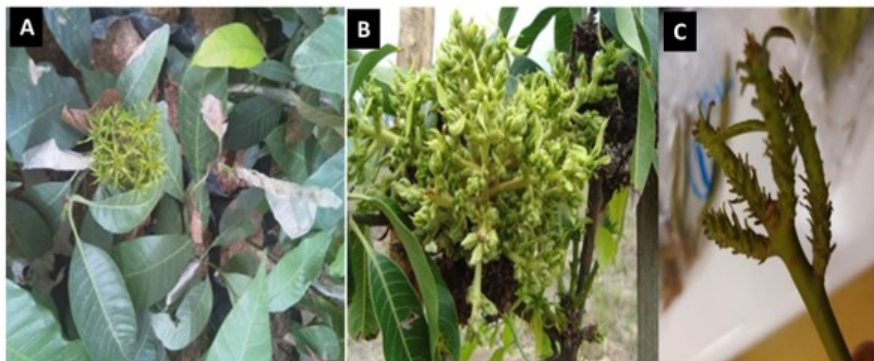


Fig 1: Naturally occurring symptoms of witches' broom (A, B), deformity (B), and shoot necrosis (C) in deformed branches observed in mango tree.

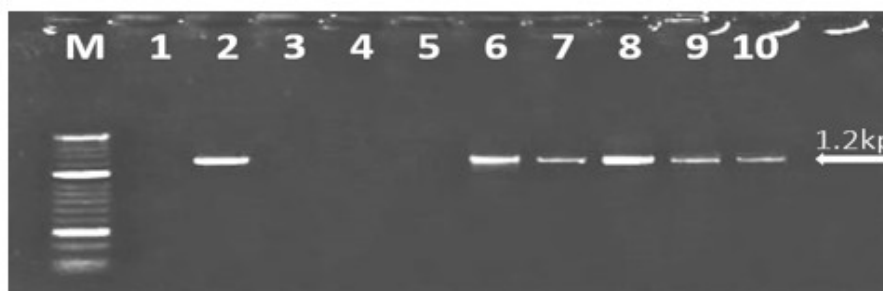


Fig 2: Agarose gel electrophoresis (1%) of PCR using primer pair P1/P7 followed by nested PCR using primer pair R16F2n and R16R2 that produced a prominent band of 1.2 kb (lanes 2,6,7,8,9,10 show visible bands). No results were found in lanes 1,3,4,5. Lane M represents the 50bp HyperLadderTM II DNA marker (Bioline Ltd, USA).



Fig 3: Agarose gel (1%) of nested PCR using fU5/rU3 primers. In lanes 1,3,4,5,6,7 visible bands appear at 880bp while lanes 2 and 9 shows no result as a negative control. Lane M represents the 50bp HyperLadderTM II DNA marker (Bioline Ltd, USA).

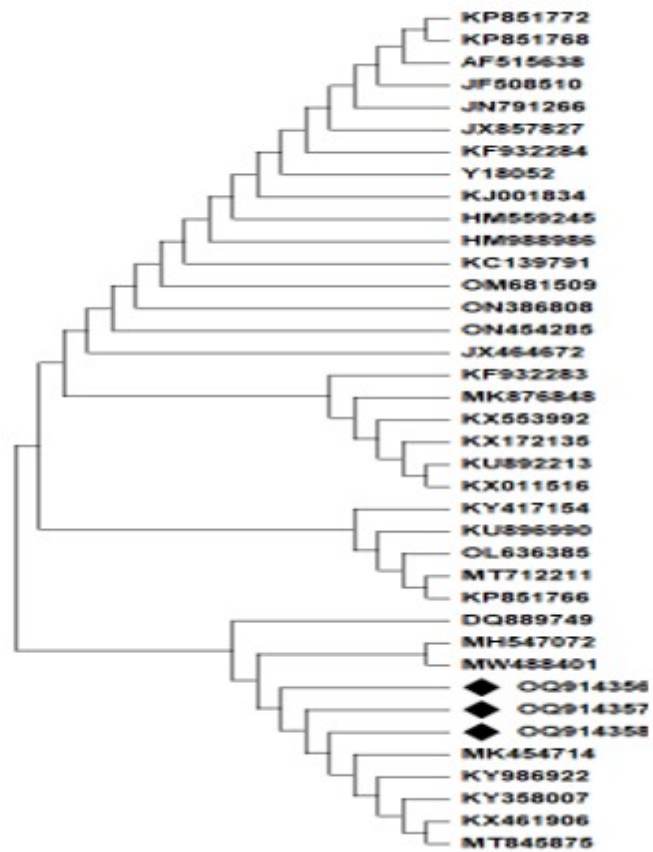


Fig 4: Phylogenetic tree shows the relationships between three phytoplasmas isolates identified from the Mango tree in Saudi Arabia and the respective isolates reported worldwide. The phylogenetic tree was created using the Maximum-Likelihood approach and 1000 bootstrap replications method.

Tables

ACC. No	Host	Country	Nucleotide percentage identity		
			OQ914356	OQ914357	OQ914358
MT845875	<i>Lactuca sativa</i>	Iran	100	100	100
KX461906	<i>Onobrychis vicifolia</i>	Iran	100	100	100
KY358007	<i>Sidens alba</i>	Iran	100	100	100
KY986922	<i>Cichorium intybus</i>	Saudi Arabia	100	100	100
MK454714	<i>Coreopsis grandiflora</i>	Iran	100	100	100
OQ914356	<i>Mangifera indica</i>	Saudi Arabia	100	100	100
OQ914357	<i>Mangifera indica</i>	Saudi Arabia	100	100	100
OQ914358	<i>Mangifera indica</i>	Saudi Arabia	100	100	100
MW488401	<i>Senecasa hiqida</i>	India	99.9	99.9	99.9
MH547072	<i>Vigna unguiculate</i>	India	99.9	99.9	99.9
DQ889749	<i>Lactuca serriola</i>	Iran	100	100	100
KP851766	<i>Malus domestica</i>	Lebanon	99.9	99.9	99.9
MT712211	<i>Conocarpus erectus</i>	Iran	99.7	99.7	99.7
OL636385	<i>Gladialis sp</i>	Italy	99.8	99.8	99.8
KU896990	<i>Osburnalis korvathi</i>	Italy	99.8	99.8	99.8
KY417154	<i>Neolitturus haematocaps</i>	Turkey	99.8	99.8	99.8
KX011516	<i>Vitis vinifera</i>	Iran	99.9	99.9	99.9
KU892213	<i>Brassica campestris</i>	Pakistan	99.9	99.9	99.9
KX172135	<i>Sophora alopecuroides</i>	Iran	99.9	99.9	99.9
KX553992	<i>Black locust</i>	Iran	99.9	99.9	99.9
MK876848	<i>Solanum melongena</i>	Turkey	99.9	99.9	99.9
KF932283	<i>Sophora alopecuroides</i>	Iran	99.9	99.9	99.9
JX464672	<i>Sesamum indicum</i>	Iran	99.9	99.9	99.9
ON454285	<i>Lactuca sativa</i>	Turkey	99.9	99.9	99.9
ON386808	<i>Catharanthus roseus</i>	Iran	99.9	99.9	99.9
OM681509	<i>Cregis foetida</i>	Turkey	99.9	99.9	99.9
KC139791	<i>Sesamum indicum</i>	Turkey	99.9	99.9	99.9
HM988986	<i>Brassica rapa</i> cv. Toria	India	99.9	99.9	99.9
HM559245	<i>Brassica rapa</i> cv. Toria	India	99.9	99.9	99.9
KJ001834	<i>Sophora alopecuroides</i>	Iran	99.9	99.9	99.9
Y18052	<i>Knautia arvensis</i>	Italy	99.9	99.9	99.9
KF932284	<i>Prunus persica</i>	Iran	99.9	99.9	99.9
JX857827	<i>Pteris achioides</i>	Italy	99.9	99.9	99.9
JN791266	<i>Catharanthus roseus</i>	Italy	99.9	99.9	99.9
JF508510	<i>Solanum lycopersicum</i>	Iran	99.9	99.9	99.9
AF515638	<i>Lactuca serriola</i>	Lebanon	99.9	99.9	99.9
KP851768	<i>Oxyris alba</i>	Lebanon	99.9	99.9	99.9
KP851772	<i>Sinapis arvensis</i>	Lebanon	99.9	99.9	99.9

Table 1: Percentage identity between three Saudi Arabian phytoplasma isolate from mango compared with other 16SrIX phytoplasma group isolates posted in the GenBank

DISCUSSION

Mango malformation and witches-broom symptoms were observed in mango orchards in the Al-Baha region, Saudi Arabia, during 2022 and were detected by PCR using P1/P7, R16F2n/R16R2, and fU5/rU3 primers. In Saudi Arabia, this is the first report of phytoplasma infection in mango trees.

Numerous phytoplasma strains have been identified as infecting mangoes. Electron microscopy and staining techniques were used to identify a phytoplasma related to deformed mango inflorescence and the sudden mortality occurrence in Egypt and Pakistan [22,23] and a 'Candidatus Phytoplasma asteris'-connected strain (16SrI group) was identified from Pakistan, infecting mangoes with deformity [24]. Additionally, a 16SrII-D phytoplasma was found in mango with malformations, and many phytoplasma groups (16SrI, 16SrII, and 16SrV) were present in mango trees exhibiting overgrowth of foliage, causing bunchy top symptoms [25,26].

It has been confirmed that a phytoplasma from Punjab, India, belonging to the 16SrI-D subgroup, causes mango malformation disease. Subgroup affiliation for the strains of the 16SrI group found in mangos has not been documented before. Different groupings of phytoplasma strains have been documented to be hosted by twelve fruit species [25,27].

Although the 16SrI-D subgroup is occasionally documented, it is known to be linked to severe infection in paulownia that exhibits symptoms similar to witches' brooms in China [28,29]. The discovery of a new host plant species belonging to the 16SrI-D subgroup reported here has important epidemiological implications.

Most Middle Eastern nations have reported several phytoplasma infections. While the 16SrIX group of phytoplasma is more common in northeast Iran, Lebanon, and Turkey, likewise, the 16SrII group of phytoplasmas is abundantly found in the Gulf States and the south of Iran [30]. The presence of the same phytoplasma disease caused by different strains in different countries could be due to differences in the vector and the phytoplasma source. In phytoplasma, there is no specific host-pathogen relation because different or specific strains of phytoplasma can be found in one plant species, although several plant species can be infected by one strain [30].

In this study, phytoplasma was detected by PCR in mango samples collected from Bisha. The sequence analysis revealed that the three Saudi phytoplasma isolates, related to the witches' broom phytoplasma, showed the highest identity (100%) with the Iranian and Saudi Arabian phytoplasma isolates.

The results of this study indicated that the symptoms of yellowing, chlorosis, and malformation in Mango plants were associated with phytoplasma disease in samples collected from Bisha, Al-Baha Region, Saudi Arabia. Tapia- Tussell et al. [31] characterized phytoplasma 16SrIII from tomato samples showing similar symptoms. Among these groups 16SrI and 16SrII groups are the most common infecting tomatoes. Nothing is recorded for 16SrII-phytoplasma group except the tomato big bud from Australia [32]. Contaldo et al. (2022) have reported up to 30% disease incidence in a commercial mango nursery in Ayodhya, Uttar Pradesh. Three phytoplasmas from the 16S rRNA gene groups 16SrI, 16SrII, and 16SrVI were identified by PCR/RFLP. Additional multigene analysis (imp, sap, rp, and clp) enabled the 16SrII strain to be thoroughly characterized and validated its association with *Candidatus Phytoplasma australasia* [33].

However, different crops like alfalfa, Giant reed (*Arundo donax*), date palm, sand olive shrub (*Dodonaea angustifolia*), tomato, cooba tree (*Acacia salicia*), some weeds, and lime have been affected by phytoplasma in different regions (Al-Hassa, Jizan, Qassim, and Al-Riyadh) of Saudi Arabia [34-38]. Multigene-based identification of phytoplasma could clarify their spread in orchards via insect vectors and agricultural practices. Restrictions on the sharing of planting materials for grafting within the country could help reduce the risk of phytoplasma infection in mango orchards.

CONFLICT OF INTEREST

The authors declare no conflicts of interest that are relevant to this article, and all authors approved the manuscript and agreed to submit it for publication.

AUTHOR CONTRIBUTIONS

Conceived and designed the study, materials preparation, data collection and analysis were performed by Mahmoud A. Amer, Muhammad Amir and Mohammed A. Al-Saleh. Analysis of data was done by Zaheer Khalid and Ibrahim M Al-Shahwan. The first drafting of the manuscript was written by Mahmoud A. Amer and Muhammad Amir.

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