Identification of two phytoplasma groups in Alwijam-affected date palms and their possible alternative hosts in Saudi Arabia

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Alhudaib K. [1], Rezk A. [2,3] and Alsaleh M. [4]

[1] Plant Protection Dept, King Faisal University, PO Box 55009 Alhassa, 31982, Saudi Arabia
[2] Biotechnology Dept, King Faisal University, PO Box 55009 Alhassa, 31982, Saudi Arabia
[3]Virology Dept, Plant Pathology Research Institute, ARC, Giza, Egypt
[4] Plant Protection Dept, King Saud University, PO Box 2460, Riyadh, 11451, Saudi Arabia

ABSTRACT

Date palm is affected by the Alwijam disease in many regions of the Kingdom of Saudi Arabia. The Alwijam phytoplasma survey was conducted in new date palm-growing areas of Saudi Arabia. Leaf samples were collected from 943 date palm plants, with and without Alwijam symptoms. The DNA was extracted from leaf midribs and indexed by a nested PCR reaction using different sets of 16S rRNA phytoplasma generic primers P1/P7 or R16mF2/R16mR1 and R16F2n/R16R2. PCR amplicons were cloned and sequenced. The consensus sequences from the Alwijam phytoplasma were submitted to GenBank and compared with those from other phytoplasma. Two phytoplasma groups were identified; 16SrI (Candidatus Phytoplasmas asteris) in Alhasa, and 16SrII (aurantifolia) in other locations of Saudi Arabia. Ocimum basilicum and Medicago sativa were found as possible alternative hosts for Alwijam 16SrII phytoplasma.

Keywords: *Candidatus Phytoplasma, Date palm, 16SrI, 16SrII, Saudi Arabia.*

INTRODUCTION

Date palm, (*Phoenix dactylifera* L.) is a major cash crop in Saudi Arabia. It is a dioecious, monocot plant species that belongs to the family Arecaceae. The area planted with date palms in Saudi Arabia spread over 156,901 hectares with over 23 million trees, with more than three million trees are grown at Alhasa Oasis. The annual date production is over 1,095,158 tons per year (Anonymous, 2014). The eastern region, on the Arabian Gulf, is one of the most important regions for date palm production for centuries. The first record of Alwijam disease dates back to 1945 (Badawi, 1945), and it was first reported in Saudi Arabia by El-Baker (1952) and Nixon (1954).

Phytoplasma has been reported to be associated with a few palm diseases, where lethal yellowing disease that affects coconut palms and date palms in the Caribbean, West Africa, Florida and Texas (Gurr *et al.,* 2016 and Harrison *et al.,* 2014); a slow decline (Arkish disease) and white tip dieback have become limiting factors in date production in Sudan (Cronje *et al.,* 2000a and b). Moreover, Alhudaib and Rezk (2014) reported a phytoplasma of group 16SrII '*Ca. P. aurantifolia'* associated with a disease in tomato plants growing near the date palm trees in Saudi Arabia, while Alkhazindar (2014) identified a '*Ca. P. asteris'* in date palm in Egypt.

Phytoplasma was first discovered in 1967 in aster plants in Japan; phytoplasma are uncultivable bacteria and obligate parasites of the phloem in the infected plants (Abdullah *et al.,* 2010 and Bertaccini and Duduk, 2009). They are mainly transmitted by insect vectors of the *Hemiptera* order, families *Cicadellidae, Fulgoroidea* and *Psyllidae* (Weintraub and Beanland, 2006). Alwijam disease is affecting palms that show symptoms yellow streaks on the leaves and stunting. A decrease in the fruit stalk and fruit size of about 30% is probably the most evident symptom of the disease. Leaves develop chlorosis and very short lifespan, where yellowing increases with the age of the palm tree and eventually leads to plant death. Diseased spathes tend to be quite shorter than healthy ones and have a tendency to split in two before complete emergence. Attempts to associate viral, fungal and nematode pathogens with the disease have so far failed (Abdusalam *et al.,* 1992 and 1993 and Elarosi *et al.,* 1982).

Previous research on Alwijam which affected date palms suggested a phytoplasma as the possible disease (Abdusalam *et al.,* 1993), further supported by (El-Zayat *et al.,* 2000). Alhudaib *et al.* (2007) identified a phytoplasma of 16SrI group, *'Candidatus Phytoplasma asteris*' associated with Alwijam disease in Alhasa (Alhudaib *et al.,* 2015). However, an extended survey was required to identify the possible occurrence of phytoplasma mixed infections in Alhasa and other date palm-growing neighboring areas in Saudi Arabia, and to assess their impact for the date palm production of fruit reduced in size (about 30%), based on varieties in the region. The objective of this study was identification of phytoplasma in date palm tree in Saudi Arabia.

MATERIALS AND METHODS

Plant material

A survey for the Alwijam phytoplasma was conducted in six regions of Saudi Arabia (Fig. 1). Nine hundred and forty three date palm leaf samples were collected from six different locations in Saudi Arabia as follows; 581 samples prospective Alwijam symptoms and 362 from date palms symptomless date palm. In addition, 189 samples of non-date palm plant species growing underneath and surrounding the date palm trees were collected and kept in plastic bags in ice boxes and assigned an ID code number.

DNA extraction

Total NA was extracted at the Pests and Plant Diseases Unit at King Faisal University, from date palms using the CTAB method (Doyle and Doyle, 1990) and non- date palm species using a combination of two methods reported by Anfoka *et al.* (2008) and Dellapota *et al.* (1983). Total DNA from a disease-free tissue culture-derived date palm was used as a negative control

PCR amplification of the 16S rRNA gene

Total DNAs were used as templates for a nested PCR (nPCR) assay with primers that amplify the phytoplasma 16S rRNA gene: P1/P7 (Deng and Hiruki, 1991 and Smart *et al.,* 1996) or R16mF2/R16mR1 (Gundersen *et al.,* 1996) for the first PCR round and R16F2n/R16R2 (Gundersen and Lee, 1996) for the nested PCR (Table 1). PCR reactions were performed in 25 µl volumes containing 10-20 ng of DNA template, 1 µl for each primer (Mecrogen, Korea) (10 pmol), 2.5 µl of 2.5 mM dNTPs (Promega, USA), 2.5 µl of 25 mM $MgCl_2$, 2.5 µl of 10 X Taq polymerase buffer and 1.5 units of Taq DNA polymerase (Invitrogen, USA). Amplifications were

obtained after 35 cycles at 94 °C for 45 seconds (94 °C for 2 min as initial denaturation), annealing at 55 °C for 1 min, extension at 72 °C for 2 min, and 10 min at 72 °C, held at 4°C. For nested PCR amplifications, the first round PCR products were 1:80 diluted and 1 µl of the dilution was used as a template. Five microliters from each nested PCR amplification were electrophoresed in a 1% agarose gel using TBE buffer, stained with ethidium bromide, and visualized by UV transillumination and photographed (Syngene Bio Imagins, IN Genius, USA). A DNA size marker (1 kb DNA marker Promega, USA) was used to estimate PCR product sizes.

Fig. (1): Locations for sampling of date palm and non-date palm in Saudi Arabia (shown as red circle).

Sequencing and data analysis

PCR amplicons of nested PCR were purified on spin columns (QIAquick gel extraction kit; QIAGEN, Germany), cloned into a pGEM-T Easy vector (Promega, USA) and sequenced bi-directionally in an ABI 377XL automated DNA sequencing instrument (Macrogen Inc., Soul, Korea). The consensus 16S rDNA sequences were compared by BLAST (Altschul *et al.,* 1990), trimmed and aligned cluster (Larkin *et al.,* 2007), and used to construct a phylogenetic tree using MEGA version 6 (Tamura *et al.,* 2013) and the Maximum Likelihood method based on the Tamura-Nei model to support the branch values (Tamura *et al.,* 2013). *Acholeplasma palmae* was used as an out-group to root the phylogenetic tree. The *i*PhyClassifier (Zhao *et al*., 2009) was used to perform sequence similarity and generate virtual restriction fragment length polymorphism (RFLP) profiles. Virtual RFLP analyses and 16S rRNA subgroup designation were performed according to the

procedures described by Wei *et al.* (2008), Zhao *et al.* (2009) for the seven isolated phytoplasma. DNA fragments were digested *in silico* with 17 distinct restriction enzymes by using virtual gel analysis *i*PhyClassifier (Zhao *et al*., 2009).

Table (1): Primers used for the amplification of the phytoplasma 16S rRNA gene.

Primer Name	Sequences 5′----------3′	Expected size band	Sources
P1	AAGAGTTTGATCCTGGCTCAGGATT		Deng and Hiruki (1991)
P7	CGTCCTTCATCGGCTCTT	1784 bp	Smart *et al.* (1996)
R16mR1	CTTAACCCCAATCATCGAC		
R16mF2	CATGCAAGTCGAACGGA	1416 bp	Gundersen *et al.* (1996)
R16F2n	GAAACGACTGCTAAGACTGG	1253 bp	Gundersen and Lee (1996)
R16R2	TGACGGGCGGTGTGTACAAACCCCG		

RESULTS

Detection of phytoplasma associated with date palm and non-date palm

A total of 943 leaf samples from date palm and 189 from non-date palm species; 17 *Amaranthus caudatus* 'cat's tail grass', 46 *Ocimum basilicum* (basil), 23 *Rubus fruticosus* ('aoluik'), *Lycopersicum spp*. (tomato), and 103 *Medicago sativa* (alfalfa') were collected. Phytoplasma was found to be associated with 15 out of 46 *O. basilicum* and 31 out of 103 *M. sativa* but not with other non-date palm samples.

Seventy one date palm leaf samples out of 943 (7.3%) PCR tested were positive for phytoplasma using the primer combination P1/P7 or R16mF2/R16mR1 for the first PCR round and R16F2n/R16R2 for the nested reaction, which yielded amplicons of the expected size (Table 2). All samples that gave positive reaction were from symptomatic date palm. The Eastern province was the province with the highest percentage of phytoplasma infection (11.97%). However, no PCR amplification was obtained for date palms symptomless from Almadinah, Hail and Jouf.

Table (2): Phytoplasma-infected date palm from each of the six locations surveyed.

Region	No. Samples	PCR results Positive	Negative	%
Almadina	141	0	141	0
Eastern P*	376	45	331	11.97
Hail	56	0	56	0
Jouf P*	50	0	50	0
Qassim	132	10	122	7.6
Riyadh	188	16	172	8.5
Total	943	71	872	7.3

*P= province

Samples of date palm tested positive by PCR and representative of different locations (from Alkharj1, Alkharj2 (90 km south east of Riyadh), Eastern province (Alhasa), Qassim and Riyadh) were sequenced and submitted to GenBank under accession numbers KY091879, KY091880, KY091878, KY091881 and KY091882, respectively.

Phylogenetic analysis

Results clearly indicated that the phytoplasma from Eastern province Alhasa (KY091878) were more different from those isolates of Riyadh, Alkharj and Qassim. The phylogenic tree presented in Fig. (2) showed that date palm phytoplasma from Eastern Province (KY091878) was clustered with those phytoplasma strains of 16SrI group in the same cluster with Aster Yellow Phytoplasma. While the phylogenic tree showed that 16S rDNA sequences of date palm phytoplasma from Alkharj (KY091879 and KY091880), Qassim (KY091881) and Riyadh (KY091882) were clustered with those phytoplasma of 16SrII group with Peanut Witches Broom phytoplasma. In addition, the phytoplasma isolated from *M. sativa* (KY091884) and *O. basilicum* (KY091883) were clustered with 16SrII group. This preliminary grouping was supported by the analysis of the evolutionary relationships (Fig 2) among those identified in this study and others from GenBank.

Virtual RFLP analyses of 16S rDNA sequences

The virtual gel RFLP analysis of 17 restriction enzyme (data not shown) could be differentiated from 30 other phytoplasma groups. Group 16SrI and 16SrII could be differentiated by analysis using the restriction enzymes *Alu*I, *Hae*III and *Hpa*II (Fig. 3). The Eastern Province Alhasa (KY091878) was different from the reference patterns of all previously established 16Sr groups/subgroups (Fig. 3). The most similar is the reference pattern of the 16Sr group I, subgroup S (GenBank accession: HM067755), with a similarity coefficient of 0.91, which is less than 0.97. The most similar was the reference pattern of the 16Sr group II, subgroup D (GenBank accession: Y10097), with similarity coefficient of 0.95, 0.94, 0.95, 0.86 and 0.95 with clones sequences of the phytoplasma from date palm Alkharj1 (KY091879), date palm Alkharj2 (KY091880), date palm Qassim (KY091881), date palm Riyadh (KY091882) and *O. basilicum* (KY091883). These similarities are less than 0.97 (Fig. 3). However the virtual RFLP pattern of the clone sequence of phytoplasma from *M. sativa* (KY091884) showed that the similarity coefficient was 0.98 with the 16Sr group II, subgroup D (GenBank accession: Y10097), which is more than 0.97.

Findings contribute to the knowledge on the biodiversity of phytoplasma associated with Alwijam disease in Saudi Arabia since

two different groups, 16SrI and 16SrII have been identified from date palms affected with Alwijam from six different locations. Although the role of either 16SrI or 16SrII groups on the occurrence of Alwijam disease in these particular regions is not clear. The fact that both groups were from a common plant host, suggest that date palm is a susceptible host to both phytoplasma groups. It is very likely that unknown epidemiology constraints may influence the spread of the disease to either new plant hosts or crops within the same regions, or new ecological niches (Lee *et al.,* 2000) in different date palm growing regions. The virtual gel RFLP analysis using the *i*PhyClassifier program revealed that the similarities of the isolated phytoplasma in this study were less than 0.97 in both cases 16SrI and 16SrII groups. So it may these strain represent as new subgroups within the 16SrI and 16SrII groups. To prove that farther more study will be needed by comparing the virtual RFLP patterns and identifying the key enzymes that will distinguish the new subgroup pattern from previously recognized group/subgroup patterns. In addition, an actual laboratory restriction digestion with the key enzyme(s) may help to confirm the new subgroup pattern. In addition, further studies are required to understand the relationships between the 16SrI and 16SrII phytoplasma affecting date palm, e.g. the factors determining their coexistence, including any epidemiological implications and impacts that geographic location and/or climate may have for the development of the Alwijam disease, and the capability of date palm to host both phytoplasma groups. A finer revision of the current crop management for date palm in Saudi Arabia is required regarding the coexistence of both groups in a common host towards recommending better strategies for a more effective disease control. The phytosanitary measures by prohibition of import of date palms from region where Alwijam disease is present, seems the only satisfactory measure recommended to prevent spread of this diseases in new region(s) in Saudi Arabia and surrounding countries.

DISCUSSION

Previous reports of phytoplasma associated with Alwijam in Saudi Arabia refer to group 16SrI identified in the Alhasa oasis in the Eastern Province (Alhudaib *et al.,* 2007). However, from the current survey, phytoplasma from two different groups: 16SrII, 'Peanut Witches Broom phytoplasma' and 16SrI, 'Aster Yellow Phytoplasma' were found in date palms affected by Alwijam in six different locations of Saudi Arabia. The group 16SrII was identified from Alkharj, Qassim and Riyadh regions; while the group 16SrI was isolated from the Eastern Province – Alhasa. The 16SrI, 'Aster Yellow Phytoplasma' is the phytoplasma group with the largest plant and insect vector host range and the most complex epidemiology (Bertaccini and Duduk, 2009 and Lee *et al.,* 2000). Results confirm that 16SrI phytoplasma are prevalent in the date palms of Alhasa and could become a threat for other non-date palms in the region. The presence of the phytoplasma group 16SrII was confirmed in date palms, basil (*O. basilicum*) and alfalfa (*M. sativa*) grown nearby the date palms in the regions of Alkharj, Qassim and Riyadh. Therefore, either basil or alfalfa may play a role as hosts for the 16SrII phytoplasma currently affecting date palms in these two neighboring regions, which may also have common polyphagous *Hemiptera* vectors. *M. sativa* is a widely known host for the 16SrII group since it has been recorded from Oman (Khan *et al., 2001 and* 2002), and Iran (Esmailzadeh *et al.,* 2011). This explains the fact that *M. sativa* scored the highest number

(103) versus (46) for *O. basilicum* hosting the 16SrII phytoplasma in both regions of Riyadh, Alkharj and Qassim.The group 16SrII, 'Peanut Witches Broom phytoplasma' has been also recorded from weeds and herbaceous crops in Africa, Asia, America, New Zealand, Southern Europe and Australia (Garnier *et al.,* 1991; Ghosh *et al.,* 1999; Leyva-Lopez *et al.,* 2002; Tessitori *et al.,* 2005; Tolu *et al.,* 2006 and Tran-Nguyen *et al.,* 2003). Particularly in the Gulf region, the group 16SrII has been identified in lime (Zreik *et al.,* 1995), sesame (Al- Sakeiti *et al.,* 2005 and Esmailzadeh-Hosseini *et al.,* 2007), garden beet (Mirzaie *et al.,* 2007) and tomato (Alhudaib and Rezk, 2014).

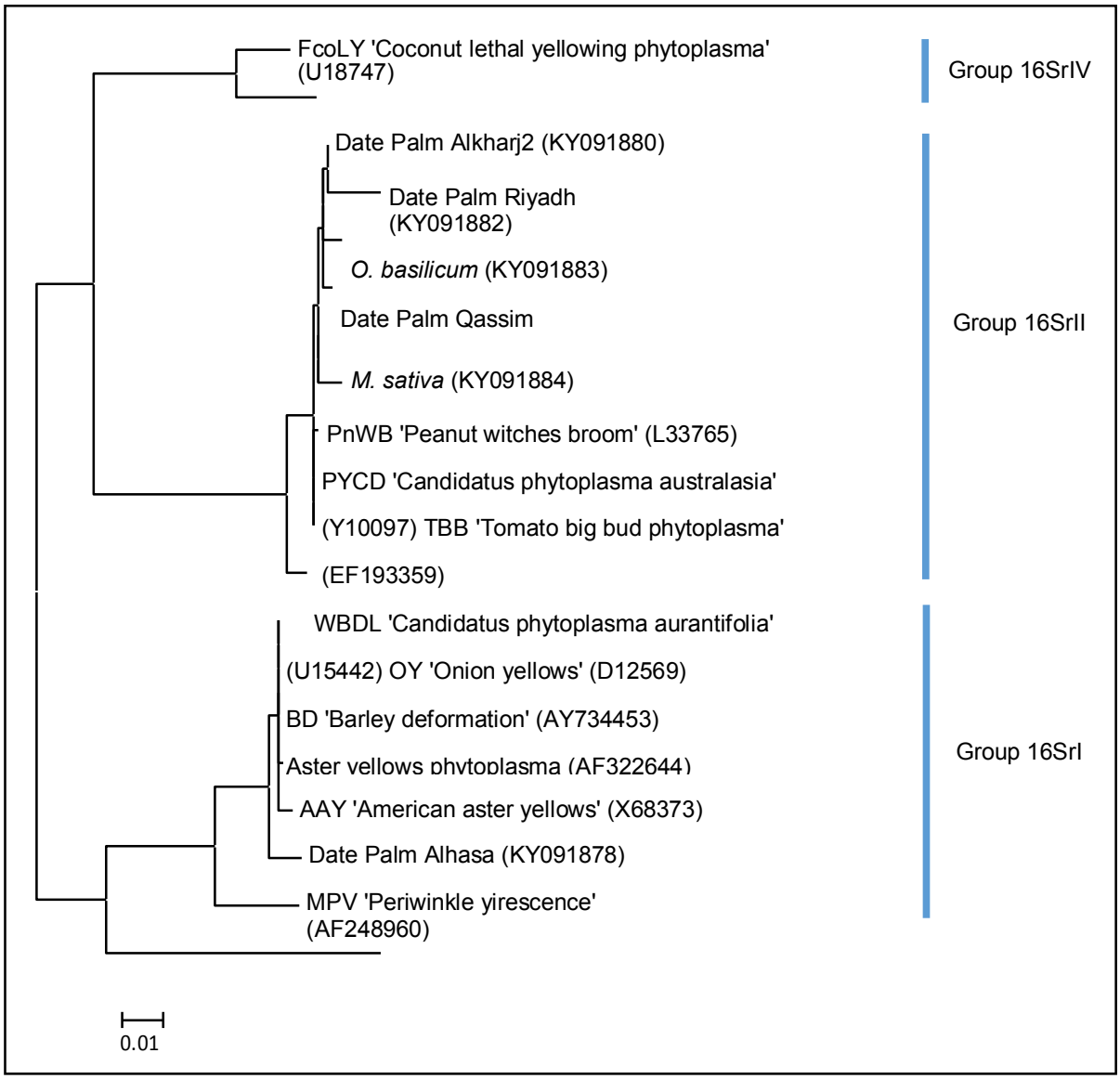

Fig. (2): Phylogenetic relationships among the Alwijam and other phytoplasma isolates identified in Saudi Arabia and those phytoplasma isolates from GenBank.

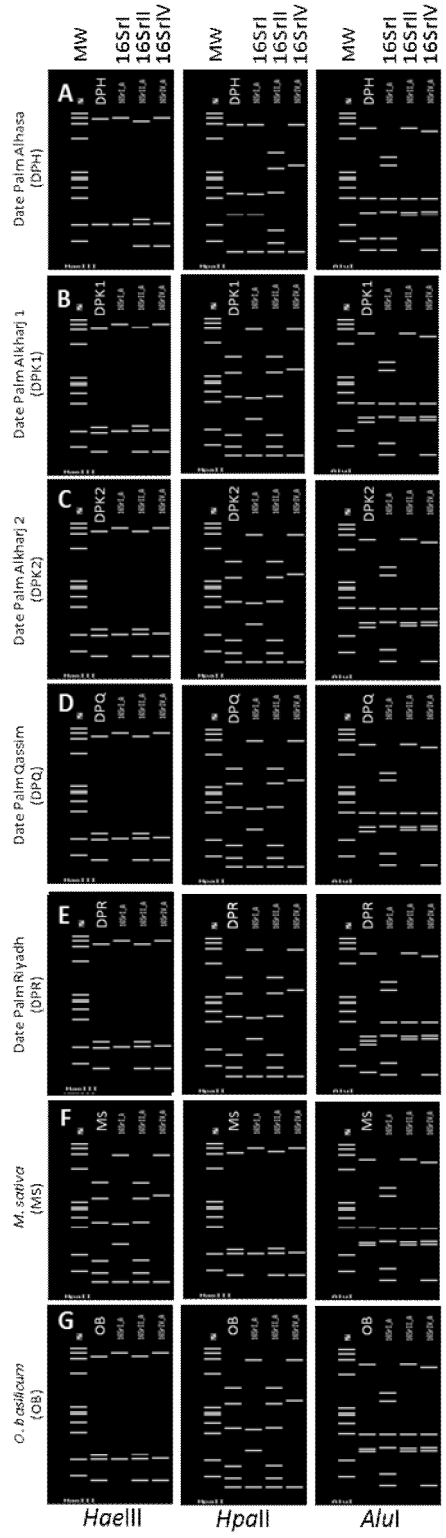

Fig. (3) :Computer-simulated virtual RFLP patterns derived from in silico digestions of phytoplasma 16S rRNA gene R16F2n/R16R2 fragments (~1.2 kb in length) from the representative seven strains with group 16SrI, 16SrII and 16SrIV, and three key enzymes: HaeIII, HpaII and AluI. MW, molecular weight marker (derived from øX174 RFI DNA); fragment sizes (bp) from top to bottom: 1353, 1078, 872, 603, 310, 281, 271, 234, 194, 118, 72

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الملخص العربي

تعريف مجموعتين من الفيتوبلازما في نخيل التمر المصاب بالوجام والعوائل البديلة في المملكة العربية السعودية

خالد عبد الله الهديب[١] ، عادل عبدالصبور رزق[٢،٣] ، محمد الصالح[٤]
[١] قسم وقاية النبات، جامعة الملك فيصل، المملكة العربية السعودية
[٢] قسم التكنولوجيا الحيوية، جامعة الملك فيصل، المملكة العربية السعودية
[٣] قسم علم الفيروسات، معهد بحوث أمراض النبات، مركز البحوث الزراعية، مصر
[٤] قسم وقاية النبات، جامعة الملك سعود، المملكة العربية السعودية

يصاب نخيل التمر في المملكة العربية السعودية بمرض الوجام في العديد من المناطق. وتشمل أعراض المرض تقزم الاوراق، خطوط صفراء، وانخفاض ملحوظ في الساق وحجم الثمار، مما يؤدي إلى الفشل في إنتاج الثمار المرغوبة وقت الحصاد. تم إجراء حصر لفيتوبلازما النخيل (الوجام) في عدد من مناطق زراعة النخيل في المملكة العربية السعودية حيث تم جمع ٩٤٣عينة ورقية من النخيل وبعض النباتات الأخرى النامية تحت أشجار النخيل بعضها يحمل اعراض المرض والبعض الاخر لا يحمل اعراض. تم استخلاص الحمض النووي من عروق الاوراق وفحصها عن طريق تفاعل PCR المزدوج (nested PCR) باستخدام مجموعات مختلفة من البادئات في منطقة 16S rRNA مثل P1 / P7 و R16mF2 / R16mR1 أو R16F2n / R16R2. تم استنساخ نواتج تفاعل PCR وتحديد تتابعاتها وايداع التتابعات لفيتوبلازما الوجام في بنك الجينات ومقارنتها مع الفيتوبلازما الاخرى. وجدت ٧١ عينة من النخيل مصابة بالفيتوبلازما من أصل ٩٤٣ (٧.٣٪) والتي تم فحصها باستخدام تفاعل PCR. انتمت العزلة من المنطقة الشرقية إلى مجموعة (‘Ca. *P. asteris*) 16SrI وهي فيتوبلازما مختلفة عن تلك المعزولة من أماكن اخرى في المملكة العربية السعودية. تم التعرف على مجموعتين من فيتوبلازما الوجام التي تصيب نخيل التمر، الاولى هي (*Candidatus Phytoplasmas asteris*) 16SrI في منطقة الإحساء بينما الأخرى هي (aurantifolia) 16SrII و ظهرت في المناطق الاخرى بالمملكة. وقد وجد ان نباتات الريحان والبرسيم الحجازى تعتبر عوائل بديلة للفيتوبلازما من مجموعة 16SrII.